Metabolic signalling and neuroendocrine stress responses in pregnancy

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Declaration

The work outlined in this thesis was undertaken in the Department of Neuroendocrinology at the University of Edinburgh, under the supervision of Professor John A. Russell. All of the work described is the original work of the author. This thesis was composed solely by the author and no part has previously been presented for degree, diploma or other qualification at this or any other university.

Juliana Bales

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For my parents and my sister

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Abstract

The hypothalamo-pituitary-adrenal (HPA) axis is hyporesponsive during late pregnancy to emotional, physical and metabolic signals. This is due to reduced responsiveness of the parvocellular paraventricular nucleus (pPVN) corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) neurones. Major adjustments in metabolic regulation during late pregnancy will help ensure a successful outcome. These include increased appetite and reduced energy expenditure during exposure to a stress, to which reduced neuroendocrine stress responses may contribute. Glucocorticoids have a key role in mobilising energy in response to acute stress, and have longer-term actions in the regulation of fat storage. I investigated the relationship between the HPA axis and endocrine factors relating to adipose tissue storage and appetite regulation during late pregnancy in rats. The responsiveness of the hypothalamic neurones regulating ACTH and hence corticosterone responses to insulin induced hypoglycaemia (IIH) and the orexigenic peptides NPY, orexin and ghrelin was investigated with particular focus on the mechanisms involved in restraining these responses during late pregnancy.

IIH increased ACTH secretion similarly in both virgin and pregnant rats. Unlike most stressors IIH did not stimulate parvocellular CRH mRNA expression in pPVN neurones but it did increase AVP mRNA expression.

The responsiveness of the HPA axis to the orexigenic peptides ghrelin, orexin and NPY given by i.c.v. injection was markedly reduced during late pregnancy. This was at least partly a result of reduced activation of the pPVN neurones, as revealed by reduced stimulation of Fos expression in the pPVN compared with virgin rats given these peptides. ACTH secretory responses were also strongly attenuated in late pregnant rats. In contrast all three orexigenic peptides increased food intake to a similar level in both virgin and pregnant rats. Thus neuroendocrine stress responses to central administration of orexin, NPY and ghrelin are absent during late pregnancy whilst ingestive behavioural responses remain intact. Changes in brain circuitry regulating appetite during late pregnancy were shown by increased Fos activation in the lateral hypothalamic area (LHA), ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH). Supraoptic and magnocellular PVN oxytocin responses to centrally administered NPY were reduced during late pregnancy.

Endogenous opioids are involved in the attenuation of HPA axis responses to orexin and NPY during late pregnancy since pre-treatment with the opioid receptor antagonist naloxone reinstated the ACTH response and restored CRH and AVP mRNA responses. Naloxone administration revealed that endogenous opioids facilitate NPY-induced feeding in both virgin and late pregnant rats, but more importantly in late pregnant rats. Naloxone restored a Fos response in the PVN and SON in response to NPY in late pregnant rats indicating that oxytocin neurone responses to NPY are suppressed by endogenous opioids.

Basal blood glucose levels were lower in late pregnant rats than in virgins. Ghrelin increased blood glucose levels similarly in both virgin and pregnant rats, whilst NPY and orexin increased blood glucose in only the virgin rats.

In conclusion, neuroendocrine stress responses to orexin, ghrelin and NPY are reduced in pregnant rats, and this was shown for orexin and NPY to be due to endogenous opioid restraint. Endogenous opioid mechanisms have opposite effects on neuroendocrine stress responses and feeding, which will enhance energy availability for the fetuses at this time. Intact HPA axis responses to IIH will ensure continued glucose supply.

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List of Abbreviations

5HT = serotonin
11βHSD1 = 11β-hydroxysteroid dehydrogenase type 1
11βHSD2 = 11β-hydroxysteroid dehydrogenase type 2
^{35}S dATP = radioactively labelled deoxyadenosine triphosphate
aCSF = artificial cerebrospinal fluid
ABC = avidin-biotin complex
ACTH = adrenocorticotropin hormone
ADP = adenosine di phosphate
ADX = adrenalectomy
AgRP = agouti related peptide
ANOVA = analysis of variance
AP = allopregnanolone
AP = area postrema
ARC = arcuate nucleus
ATP = adenosine triphosphate
AVP = arginine vasopressin
BnST = bed nucleus of the stria terminalis
cAMP = cyclic adenosine monophosphate
CART = cocaine amphetamine related peptide
CCK = cholecystokinin
CINK = cytokine-induced neutrophil chemoattractant
CNS = central nervous system
CREB = cyclic AMP response element binding protein
CRH = corticotrophin releasing hormone
CRH-BP = CRH binding protein
CRH-R1 = CRH receptor 1
CRH-R2 = CRH receptor 2
CVO = circumventricular organ
ddH_2O = double distilled water
DAB = diaminobenzidine
DAG = diacylglycerol
DEPC = diethyl pyrocarbonate
DNA = deoxyribonucleic acid
DMH = dorsomedial hypothalamus
DTT = dithiothreitol
EB = elution buffer
EDTA = ethylene diamine tetra acetic acid
GH = growth hormone
GHRH = growth hormone releasing hormone
GHS-R = growth hormone secretagogue receptor
GLP-1 = glucagon like peptide 1
GI = gastrointestinal
GR = glucocorticoid receptor
GR = glucose responsive

GS = glucose sensitive
hnRNA = heteronuclear ribonucleic acid
HCL = hydrochloric acid
HPA = hypothalamo-pituitary-adrenal
HP = hypothalamo-pituitary
i.c.v. = intracerebroventricular
i.v. = intravenous
IIH = insulin induced hypoglycaemia
IHC = immunohistochemistry
IP_3 = inositol triphosphate
IRS = insulin receptor substrate
ISH = *in-situ* hybridisation
IL-1β = interleukin-1-beta
LHA = lateral hypothalamic area
mm = millimetre
ME = median eminence
mPVN = magnocellular paraventricular nucleus
mRNA = messenger RNA
MPo = medial preoptic nucleus
MBH = medial basal hypothalamus
MCH = melanosyte concentrating hormone
MSH = melanocyte stimulating hormone
MC3R = melanocortin 3
MC4R = melanocortin 4
MR = mineralocorticoid receptor
Na^+-K^+-ATP pump = sodium potassium ATP pump
Na_2HPO_4 = di-sodium hydrogen orthophosphate
$Na_2HPO_4.2H_2O$ = sodium hydrogen orthophosphate 2-hydrate
NaCl = sodium chloride
NaPPI = sodium pyrophosphate
NGF1-B = nerve growth factor – 1-B
NMDA = n methyl D aspartate
NPY = neuropeptide Y
NTS = nucleus of the solitary tract
O_2 = oxygen
OXM = oxyntomodulin
OVLT = organum vasculosum of the lamina terminalis
OX1-R = orexin receptor 1
OX2-R = orexin receptor 2
PI3-K = phosphatidylinositol 3 kinase
PB = phosphate buffer
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PFA = paraformaldehyde
PKC = protein kinase C
PLC = phospholipase C
POMC = proopiomelanocortin
PP = pancreatic polypeptide

PRL = prolactin
PTH = parathyroid hormone
PYY = peptide YY
pPVN = parvocellular paraventricular nucleus
RIA = radioimmunoassay
RNA = ribonucleic acid
RT-PCR = real time polymerase chain reaction
SCN = suprachiasmatic nucleus
SEM = standard error of the mean
SFO = subfornical region
SON = supraoptic nucleus
SS = somatostatin
SSC = sodium citrate
TdT0 = deoxynucleotidyl transferase
TEA = triethanolamine
Tris = trizma base
VIP = vasoactive intestinal peptide
VMH = ventromedial hypothalamus
w/v = weight/ volume
yeast tNRA = yeast transfer RNA

GENERAL INTRODUCTION

1.1 General Introduction - Stress

When faced with excessive demands or threats, a subject's adaptive responses attain a non-specific nature, known as "stress" (Habib *et al,* 2000). Hans Selye defined stress as the non-specific response of the body to a demand (Selye, 1949). Stress is defined as the state in which the brain interprets the quantity of stimulation as excessive or its quality as threatening (Chrousos *et al,* 1992). During a stress response, cardiac output and respiration are enhanced and blood flow is redirected to provide the highest perfusion to the brain and muscular system. The brain focuses on the perceived threat and acts accordingly (Habib *et al,* 2000). Endocrine mechanisms of pleasure, growth and reproduction are shut down to save energy, catabolism is increased and fuel is used to supply the brain, heart and muscles (Chrousos *et al,* 1992). Metabolic changes that occur during stress involve secretion of glucocorticoids from the adrenal cortex and secretion of adrenaline and noradrenaline by the adrenal medulla and sympathetic nerves respectively. Both adrenaline and noradrenaline have been associated with the "fight or flight response" described by Cannon in 1914 (Cannon 1930).

1.2 The "fight or flight response"

The fight or flight response was first described in 1914. The theory states that animals react to threats with a general discharge of the sympathetic nervous system (Cannon 1930). In other words, an animal has two options when faced with danger. They can either face the threat ("fight"), or they can avoid the threat ("flight"). The onset of a stress response is associated with specific physiological actions in the sympathetic nervous system, both directly and indirectly through the release of

adrenaline and to a lesser extent noradrenaline from the medulla of the adrenal glands (Wurtman *et al,* 2002). The release is triggered by acetylcholine released from preganglionic sympathetic nerves (Wurtman *et al,* 2002). These catecholamine hormones facilitate immediate physical reactions by triggering increases in heart rate and breathing, constricting blood vessels in many parts of the body—but not in muscles (vasodilation), brain, lungs and heart—and tightening muscles (Habib *et al,* 2000). An abundance of catecholamines at neuroreceptor sites facilitates reliance on spontaneous or intuitive behaviors often related to combat or escape. Normally, when a person is in a serene, unstimulated state, the "firing" of neurons in the locus coeruleus is minimal. A novel stimulus, once perceived, is relayed from the sensory cortex of the brain through the thalamus to the brain stem (for an overview of the relevant brain anatomy see Fig 1.1). This route of signalling increases the rate of noradrenergic activity in the locus coeruleus, and the person becomes alert and attentive to the environment (Wurtman et al, 2002). If a stimulus is perceived as a threat, a more intense and prolonged discharge of the locus coeruleus activates the sympathetic division of the autonomic nervous system (Thase *et al,* 1995). The activation of the sympathetic nervous system leads to the release of noradrenaline from nerve endings acting on the heart, blood vessels, respiratory centers, and other sites (Habib *et al,* 2000). The ensuing physiological changes constitute a major part of the acute stress response. The other major player in the stress response is the hypothalamic-pituitary-adrenal axis.

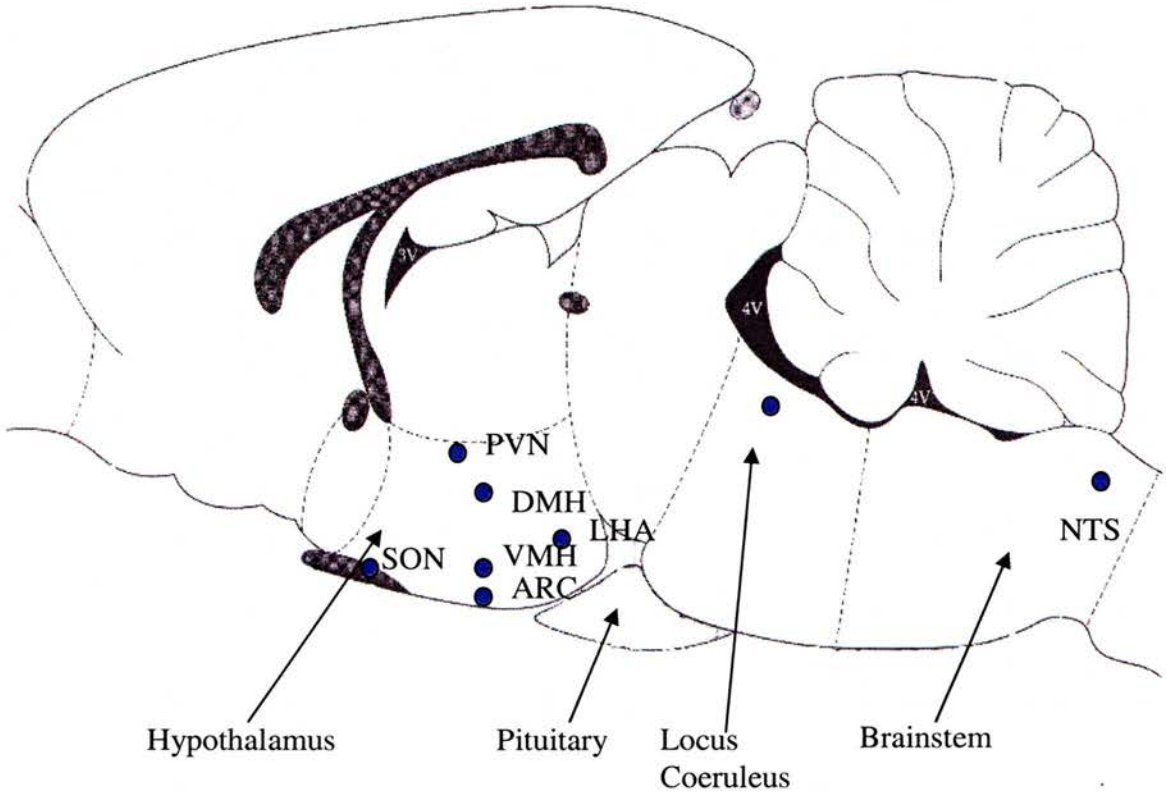


Figure 1.1: A sagittal section of the rat brain. PVN = paraventricular nucleus, DMH = dorsomedial hypothalamus, SON = supraoptic nucleus, VMH = ventromedial hypothalamus, ARC = arcuate nucleus, LHA = lateral hypothalamic area, NTS = nucleus of the solitary tract; 3V = 3rd ventricle, 4V = 4th ventricle

1.3. The components of the HPA-axis

1.3.1 The hypothalamus

The hypothalamus is a region of the mammalian brain located below the thalamus, forming the major portion of the ventral region of the diencephalon and functioning to regulate certain metabolic processes and other autonomic activities (Jiang *et al,* 2003). The hypothalamus links the nervous system to the endocrine system by synthesising and secreting neurohormones, often called releasing hormones, as needed that control the secretion of hormones from the anterior pituitary gland (Guillemin, 1972). The hypothalamus is a very complex region, and even small nuclei within the hypothalamus are involved in many different functions. The paraventricular nucleus (PVN) for instance contains oxytocin and vasopressin neurons which project to the posterior pituitary, but also contains neurones, producing CRH, that regulate adrenocorticotropin (ACTH) secretion (Koutcherov *et al,* 2000).

The PVN (see Appendix B) is an aggregation of neurones in the hypothalamus, adjacent to the third ventricle. The PVN is highly vascularised, but is inside the blood-brain barrier, although the neuroendocrine neurons in this nucleus project to sites (the median eminence and the posterior pituitary) that lack a blood-brain barrier (Armstrong & Hatton, 1980). The PVN contains magnocellular neurosecretory cells whose axons extend into the posterior pituitary, parvocellular neurosecretory cells that project to the median eminence, and several populations of peptide-containing cells that project to many different brain regions (Armstrong & Hatton, 1980).

The parvocellular PVN (pPVN)

The parvocellular neurosecretory neurons of the PVN project axons to the median eminence, at the base of the brain (Swanson & Sawchenko, 1980). At the median eminence, the neurosecretory nerve terminals release peptides into the blood vessels of the hypothalamo-pituitary portal system. These vessels carry the peptides to the anterior pituitary gland, where they regulate hormone secretion into the systemic circulation (Swanson & Sawchenko, 1980). The parvocellular neurosecretory cells include: cells that synthesise corticotropin releasing hormone (CRH), which regulates ACTH secretion from the anterior pituitary gland, cells that make arginine vasopressin (AVP): AVP released from these neurons also regulates ACTH secretion; CRH and AVP act synergistically to stimulate ACTH secretion (Gillies & Lowry, 1979). AVP has little ACTH secretagogue activity alone. In non-stressful situations, CRH and AVP are secreted in the portal system in a circadian and pulsatile fashion (Chrousos & Gold, 1992). The amplitude of these CRH and AVP pulses increase in the early light phase in humans; this results in an increase of ACTH and cortisol in the general circulation. The circadian release of CRH, AVP, ACTH and corticosterone is controlled by the suprachiasmatic nucleus (SCN) (Takahashi *et al,* 2001). These circadian variations are disrupted by stress.

The magnocellular PVN (mPVN)

The magnocellular cells in the PVN produce oxytocin and vasopressin (Sokol *et al,* 1976). These peptide hormones are packaged in large dense-core vesicles, which are transported down the axons and released from neurosecretory nerve terminals in the posterior pituitary gland. Similar magnocellular neurons are found in the supraoptic

nucleus (SON) (Reaves & Hayward, 1979). In the rat, oxytocin secretion is increased by several types of stressors (Lang *et al,* 1983; Gibbs 1984).

1.3.2. The pituitary gland

The pituitary gland is located at the base of the brain. It consists of two parts; the anterior pituitary and the posterior pituitary.

The anterior pituitary

The anterior pituitary (also called the adenohypophysis) is the anterior lobe of the pituitary gland. Under the influence of the hypothalamus via releasing factors secreted into the hypothalamo-hypophyseal portal system the anterior pituitary produces and secretes several peptide hormones that can regulate many physiological processes including stress, growth and reproduction (Thorner *et al,* 1995). Blood to the anterior pituitary is supplied by the superior hypophyseal artery, via the primary and secondary portal capillary vessels in the median eminence and anterior pituitary respectively and it secretes hormones into capillaries that drain into the hypophyseal vein (Bergland *et al,* 1977).

The posterior pituitary

The posterior pituitary (also called the neurohypophysis) is the posterior lobe of the pituitary gland. The posterior pituitary consists mainly of neuronal projections extending from the SON and the magnocellular PVN (mPVN) in the hypothalamus (Zimmerman *et al,* 1984). The posterior pituitary receives its blood supply from the inferior hypophyseal artery and secretes hormones into capillaries that drain into the hypophyseal vein. These hormones are all made in the hypothalamus and transported in axons to the posterior pituitary where they are stored (Choy & Watkins, 1977).

1.3.3. The adrenal gland

The adrenal glands are located in the abdomen above and adjacent to the kidneys. The adrenal gland is separated into two distinct structures, the adrenal medulla and the adrenal cortex. The adrenal medulla is at the centre of the adrenal gland surrounded by the adrenal cortex. The adrenal medulla is the body's main source of the catecholamine hormone adrenaline and also produces noradrenaline, which is also released by sympathetic nerve terminals. Some cells of the adrenal cortex belong to the HPA axis and are the source of glucocorticoid hormones.

The adrenal medulla

The adrenal medulla is the principal site of the conversion of the amino acid tyrosine into adrenaline and also produces noradrenaline (Seidi & Unsicker, 1989). Physiological effects of adrenaline and noradrenaline include increased heart rate, blood vessel constriction, bronchiole dilation and increased metabolism, all of these are characteristic of the "fight or flight" response (Cannon *et al,* 1914).

The adrenal cortex

The adrenal cortex produces mineralocorticoids and gluococorticoids including aldosterone and corticosterone respectively. The adrenal cortex is divided into three distinct layers; the zona glomerulosa which produces mineralocorticoids (e.g. aldosterone) and the zona fasiculata which chiefly produces glucocorticoids. The final layer of the adrenal cortex is the zona reticularis, which is the principal source of glucocorticoids with the fasiculata becoming activated after prolonged stimulation (Ricciardi *et al,* 1984). Glucocorticoid, but not mineralocorticoid secretion is controlled by and dependent upon ACTH. Corticosterone increases metabolism in

several ways; stimulating the release of amino acids, breaking down fat to stimulate gluconeogenesis, producing glucose from newly released amino acids and conserving glucose by inhibiting uptake into muscle and fat cells (van de Kar *et al,* 1981).

1.4. Neurocircuitry of stress: Pathways regulating HPA axis

Because excess glucocorticoid can cause detrimental effects it is crucial that the size and duration of the stress response is limited. This control is accomplished by a "glucocorticoid negative feedback" loop where glucocorticoids themselves can control their own release (Keller-Wood & Dallman, 1984). The parvocellular CRH/AVP neurones in the PVN play a key role in the stress response (Antoni, 1986); these neurones integrate excitatory and inhibitory impulses from a wide variety of neuronal inputs into a signal to increase ACTH secretion. CRH is the main regulator of ACTH secretion (Orth, 1982), but AVP is also released into the hypothalamo-portal system where it stimulates release of ACTH (Da Costa *et al,* 1996).

1.4.1. Excitatory stress pathways

Three different groups of afferents- an ascending brainstem pathway, the circumventricular organs and hypothalamic and basal forebrain pathways- can activate the CRH and AVP neurones in the pPVN. The combined properties of these three groups, dictates the strength of individual stressors (Zeigler & Herman, 2002).

Brainstem pathways

Activation of the brainstem is needed for autonomic activation, somatosensory pain processing and controlling arousal. It also plays a key role in activating the HPA axis. The medial part of the pPVN receives information directly from catecholaminergic and non-catecholaminergic regions of the nucleus of the solitary tract (NTS) (Cunningham & Sawchenko, 1988). Information sent to the pPVN from catecholaminergic regions of the NTS is critical for HPA axis activation following stress (Plotsky *et al,* 1989) and damage to the ascending noradrenergic pathways also reduces HPA activation following stress (Szarferczyk *et al,* 1987). The ascending noradrenergic pathways affect HPA responsiveness differently depending on the nature of the stressful stimuli (Herman *et al,* 2002). The A2 region of the NTS is known to relay information directly to the PVN (de Horst *et al,* 1989) and can quickly initiate gluocorticoid responses to and in defense of cardiovascular collapse. The locus coeruleus is the source of another ascending noradrenergic pathway which plays a key role in activation of the HPA axis, it does not however project to the PVN indicating that its role in activating the HPA axis may be via interneurons (Cunningham & Sawchenko, 1988). Both serotonin and acetylcholine have excitatory effects on the HPA axis and both increase CRH and corticosterone release (Jones & Gillham, 1988). Ascending pain pathways are also thought to provide a stimulatory input to the HPA axis, these pathways are thought to act via the ventrolateral medullary A1 noradrenergic neurones or through the A6 region of the locus coeruleus (Palkovits *et al,* 1999).

The circumventricular organs

The circumventricular organs are midline structures bordering the 3rd and 4th ventricles. They are unique areas of the brain outside the blood brain barrier and are recognised as important sites for communicating with the cerebrospinal fluid (CSF) and between the brain and peripheral organs via blood-borne products. CVO's include the pineal gland, median eminence (ME), subfornical organ (SFO), the area postrema (AP), the subcommissural organ and the organum vasculosum of the lamina terminalis (OVLT).

The medial part of the pPVN receives direct projections from the CVO's including from the SFO and the OVLT. Projections from the SFO to the pPVN increase CRH secretion (Lind *et al,* 1984). The SFO is an important component of a system of neurones that surrounds the OVLT and the medial preoptic nucleus. This collection of neurones has direct projections to the magnocellular secretory system as well as to the parvocellular system and serves as a link between the HPA axis and the neurohypophyseal system (Lind *et al,* 1984). Damage to the area postrema impairs ACTH secretion following stress (Lee *et al,* 1998) though it does not have substantial direct projections to the PVN (Oldfield & McKinley, 1994).

Hypothalamic and basal forebrain pathways

Local projections comprise the majority of inputs into the pPVN. This includes inputs from the dorsomedial hypothalamus (DMH), medial preoptic area (MPo), anteroventral third ventricular region, ventromedial hypothalamus (VMH) and the posterior division of the bed nucleus of the stria terminalis (BnST) (Zeigler *et al*, 2002). The PVN also receives projections from cells immediately surrounding it in the subparaventricular zone and the perifornical area (Sawchenko & Swanson, 1983).

Projections from these areas communicate with the PVN mediating both excitation and inhibition of the HPA axis. Inhibitory GABAergic projections are positioned to supply inhibition of basal and stress-induced HPA activity (Cullinan *et al,* 1993). Local circuit excitation may be initiated by glutamatergic afferents (Herman *et al,* 2000).

1.4.2. Inhibitory stress pathways

Inhibition of the HPA axis is mediated by humoral (glucocorticoid mediated) and neuronal (glucocorticoid independent) pathways (Keller-Wood & Dallman, 1984). The humoral part comprises a "glucocorticoid negative feedback" loop. Glucocorticoids were originally assumed to exert delayed effects via a steroid receptor-mediated action, however glucocorticoids can also inhibit ACTH secretion within minutes (fast feedback) (Keller-Wood & Dallman, 1984). However neural inhibitory pathways to the PVN can function in the absence of glucocorticoids (Keller-Wood & Dallman, 1984).

1.4.2.1. Glucocorticoid negative feedback

Glucocorticoids play a key role in regulating basal activity of the HPA axis and in termination of the stress response. They act on hypothalamic regulatory centres such as the hippocampus, frontal cortex, hypothalamus and pituitary gland (Chrousos, 2000). The inhibitory glucocorticoid feedback on the ACTH secretory response limits the duration of total tissue exposure to glucocorticoids minimising the detrimental effects of these hormones.

1.4.2.2. Different types of feedback

There are three major types of glucocorticoid feedback with respect to timeframe of action: fast, intermediate and slow. These involve three separate mechanisms of corticosteroid action. Fast feedback does not require protein synthesis to occur (Keller-Wood & Dallman, 1984). The action of CRH in stimulating ACTH release is thought to be inhibited by a rapid effect of corticosteroids acting directly on the corticotroph cell membrane (Keller-Wood & Dallman, 1984). Only stimulated but not basal CRH and ACTH release are inhibited *in vitro* so it is possible that corticosterone may inhibit an event early on in stimulus-secretion coupling (e.g. cAMP production) (Hyde *et al,* 2003). In contrast, evidence to suggest that inhibition of ACTH secretion by glucocorticoid is controlled by either cAMP or cAMP-dependent phosphorylation has been found (Shipston *et al,* 1992). CRH acts via cAMP to exert short (Shipston *et al,* 1992) and long term control (Shepperd *et al,* 1991) over glucocorticoid action at the pituitary.

Intermediate feedback also decreases ACTH release in response to stimulation of the corticotroph and does not affect synthesis of ACTH (Keller-Wood, 1989); it does however appear to affect CRH synthesis and release (Keller-Wood, 1989). It requires the presence of a protein whose synthesis is corticosterone-dependent although the role of this protein is unknown (Keller-Wood & Dallman, 1984). Intermediate feedback just like fast feedback does not inhibit basal ACTH secretion in vitro (Boscaret *et al,* 1990).

Slow feedback involves a steroidal mechanism of action reducing pituitary ACTH content by decreasing levels of POMC mRNA (coding for the ACTH precursor molecule) (Keller-Wood & Dallman, 1984). Consequently, slow feedback unlike

intermediate and fast feedback inhibits both basal and stimulus-induced ACTH secretion (Hinz & Hirschellman, 2000). Corticosteroid-induced inhibition of basal ACTH secretion has been shown to occur within two hours *in vivo*; this is different from the effects on stimulus-induced secretion, suggesting that basal secretion is activated by different pathways in the brain to CRH and hence ACTH secretion (Dayanithi & Antoni, 1989).

1.4.2.3. Glucocorticoid receptors

Glucocorticoids predominantly exert their effects through ubiquitously distributed intracellular receptors. The non-activated glucocorticoid receptor resides in the cytosol in the form of a hetero-oligomer. On ligand binding the glucocorticoid receptors dissociate from the rest of the hetero-oligomer and translocate into the nucleus where they then interact with specific glucocorticoid responsive genes. The activated receptors also inhibit several transcription factors such as *c-jun*, *c-fos* and NF-κB (van der Saag *et al,* 1996).

A dual receptor system exists for glucocorticoids in the CNS consisting of the glucocorticoid receptor type I also known as the mineralocorticoid receptor which responds to low levels of glucocorticoids, and the classic glucocorticoid receptor type II which predominantly responds after stress (Reul et al, 1985). The type II receptor participates in the negative feedback control of the HPA axis. Type I receptors bind aldosterone, corticosterone, cortisol and deoxycorticosterone (Luttge *et al,* 1989). They have restricted distribution in the brain and are expressed in high levels in the hippocampus, lateral septum, dentate gyrus and brainstem (Reul & De Kloet, 1985). Type II receptors demonstrate high affinity for synthetic

glucocorticoids (e.g. dexamethasone) and lower affinity for physiological glucocorticoids (e.g. cortisol and corticosterone) (Reul & De Kloet, 1985). Type II receptors provide a mechanism by which negative feedback can terminate stress-activated neural and endocrine (HPA) activation and regulate behavioural responses to stress (McEwen *et al,* 1988). Type II receptors are concentrated in the hippocampus, septum, amygdala, PVN, cortex, brainstem and in the pituitary (Evans & Ariza, 1989).

1.4.2.4. Neuronal feedback

The HPA axis can also be inhibited independently of glucocorticoids. Lesions of selective hypothalamic nuclei can enhance the HPA axis response to various stressors (Herman & Cullinan, 1997).

GABA

The neurotransmitter GABA inhibits ACTH release (Makara & Stark, 1974). GABAergic neurones project from the BnSt, MPo and from within the hypothalamus itself directly to the PVN. A potential role for GABA is also thought to maybe exist in controlling HPA axis activity by working through neurones in the hippocampus (Herman & Cullinan, 1997).

Opioids

The HPA axis is also tonically inhibited by endogenous opioids, as shown by injection of an opioid receptor antagonist (Grossman & Besser, 1992). Opioid inhibition can be reversed with the α1-adrenoreceptor thymoxamine (Grossman & Besser, 1982) so it is thought that opioids may control the HPA axis through central noradrenergic pathways (Holsboer *et al,* 1988).

Other factors

Atrial natriuretic peptide (ANP) has been reported to inhibit the release of CRH and AVP stimulated ACTH release from pituitary cells (Antoni & Dayanithi, 1990). Somatostatin also inhibits ACTH and CRH release *in vitro* (Tizabi & Calogero, 1992). Oxytocin has also been shown to inhibit the HPA axis in women during lactation (Lightman & Young, 1989), however it stimulates ACTH secretion in the rat, provided CRH is present (Antoni *et al,* 1988). Other compounds such as adrenomedullin, thymosin-α-1, pre-pro-thyrotrophin-releasing hormone, endothelin-1, lipocortin, substance P, leptin, nitric oxide and carbon monoxide (Jessop, 1999) have all been reported to inhibit the HPA axis in rats. Exogenous cannabinoids can also exert robust effects on hormone secretion from the pituitary inhibiting neuroendocrine function suppressing pituitary hormone release, and in particular inhibit CRH neurons (Tasker, 2004).

1.5. Stressors

Individuals can experience either internal or external stressors. Internal stressors can be physical or psychological. An example of a psychological stressor is intense worry about a harmful event. The neural circuits mediating neuroendocrine stress responses to psychological stressors involve cortical activation of the basolateral amygdala, which in turn activates the central nucleus of the amygdala. The central nucleus then activates hypothalamic neurones directly and indirectly through the BnST (Le Doux, 1995); it may also activate circuits involving brainstem serotonergic and catecholaminergic neurones (Van de Kar *et al,* 1999).

A physical stressor has a direct effect on the body; this may be an external environmental condition (e.g. heat, cold and noise) or internal physiological demands on the body (e.g. cardiovascular, osmotic and immune challenge or hypoxia). These stressors are all relayed directly to the PVN by visceral afferent pathways and represent a direct threat to survival (Dayas & Day, 2002). In these situations it is advantageous to rapidly relay information directly to the PVN.

Stressors can also be defined as those that arise suddenly over a short period of time, i.e. acute stressors, or those that develop over a longer period of time and constantly recur, i.e. chronic stressors (Kennedy *et al,* 1988).

1.5.1. Acute stress

Acute stress is the state of reaction to an immediate threat, preparing for what is commonly known as the "fight or flight" response. Common experimentally used acute stressors include cold, ether, intra-peritoneal hypertonic saline, restraint, forced swimming, emotional stressors and infection. All of these lead to an increase in ACTH and corticosterone secretion. Normally, once the threat has passed, the response becomes inactivated and levels of stress hormones return to normal. At a hypothalamic level acute stress causes a rapid increase in CRH in the median eminence (Murakami *et al,* 1989), which increase reflects increased transport of CRH. The median eminence content of CRH begins to decline around 15 minutes following the onset of acute stress (Moldow *et al,* 1987). A second increase in CRH release occurs around 60 minutes after the onset of stress (Moldow *et al,* 1987); this is likely from increased CRH synthesis. Acute stress also increases AVP production. AVP is co-produced with CRH and is secreted into the hypophyseal portal blood

from where it stimulates corticotroph activity (Antoni, 1993). Increased CRH and AVP mRNA expression in the pPVN begins to increase two hours after exposure to an acute stress and peak expression is observed 4 hours after the stressor (Ma & Lightman, 1998).

A range of intracellular transcription factors can also be seen activated in the pPVN after exposure to acute stressors. The normally quiescent immediate early gene *c-fos* is seen to be expressed in CRH and AVP containing cells in the pPVN in response to a variety of acute stressors (Hoffman *et al,* 1993). The functional relationship between *c-fos* and CRH gene regulation may not be straightforward and CRH may not be under regulation of *c-fos* (Chalmers *et al,* 1995). Other transcription factors in the PVN could also modulate CRH expression (Ehlers, 1986).

Increased POMC mRNA expression (providing the precursor for ACTH) in corticotrophs has also been shown following administration of an acute stressor (Harbuz & Lightman, 1989).

In summary, acute stress results in increased release of CRH and AVP from the median eminence with rapid stimulation of gene transcription for these peptides in the pPVN. CRH and AVP cause increased release of ACTH from the anterior pituitary, with stimulation of POMC gene expression in the corticotrophs, and ACTH secretion results in increased corticosterone secretion from the adrenal gland.

1.5.2. Chronic stress

In chronically stressed animals activation of the HPA axis in response to a stressor may be reduced with each exposure until eventually there is no longer a response (Gomez *et al,* 1996). This desensitisation of HPA responsiveness to a repeated stress

may be due to desensitisation of the pituitary and down regulation of pituitary CRH receptors.

It has been shown in repeatedly restrained rats that pPVN neurones fail to respond to a single episode of restraint stress, but there are increased AVP stores in the median eminence (Ma *et al,* 1997). Basal AVP mRNA expression in the pPVN is increased during chronic stress whilst basal CRH mRNA expression is reduced (Herman, 1995). Similarly AVP mRNA expression in response to repeated stress is maintained or even sometimes increased whilst CRH mRNA remains reduced (Aguilera & Rabadhan-Diehl, 2002). Co-expression of AVP with CRH in pPVN neurones increases (Lightman & Young, 1987). The difference between CRH and AVP gene expression under chronic stress is mediated by the action of glucocorticoids, which selectively enhance vasopressin responses (Kovacs *et al,* 2000)

1.6. Corticotropin releasing hormone

After years of research the hypothalamic factor that stimulates ACTH secretion was identified as a 41-amino acid peptide which was named corticotropin releasing hormone (CRH) (Vale *et al,* 1981). CRH acts as the main regulator of ACTH secretion (Orth, 1982). I.c.v. administration of CRH produces a stress response and administration of a CRH antagonist suppresses this response (Habib *et al,* 2000). CRH type I receptor (CRH-R1) knock-out mice have a markedly deficient ability to mount a stress response (Smith *et al,* 1998). CRH has also been implicated in other components associated with the stress response such as arousal (Shibasaki *et al,* 1993) and autonomic activation (Brown *et al,* 1985).

1.6.1. Distribution of CRH neurones

Several hypothalamic nuclei contain CRH neuron cell bodies, including the MPo, DMH, ARC, posterior hypothalamus and the mammillary nuclei (Habib *et al,* 2000). The PVN of the hypothalamus contains the majority of CRH cell bodies that stimulate ACTH secretion (Habib *et al,* 2000). These CRH neurones originate in the pPVN and send axon terminals to the capillaries of the median eminence (Habib *et al,* 2000). CRH is also present in a small group of neurones in the PVN that project to the brainstem and the spinal cord. These neurones regulate the autonomic nervous system (Turnbull *et al,* 1997).

CRH cell bodies are also found in the central nucleus of the amygdala, the substantia innominata and the BnST (Cummings *et al,* 1983). CRH neurones in the central nucleus of the amygdala project to the PVN and to the brainstem (Weidenfeld *et al,* 2002), these projections may account for neuroendocrine, autonomic and behavioural effects of CRH (Weidenfeld *et al,* 2002). The CRH neurones in the BnST project to the parabrachial nuclei and dorsal vagal complex in the brainstem to coordinate autonomic activity (Grigoriadis *et al,* 1993).

CRH interneurones have been seen in the second and third layers of the cerebral cortex and project to layers I and IV (Palkovits *et al*, 1985), these may be very important for the behavioural actions of the peptide. Scattered CRH cells have also been seen in the deeper layers of the neocortex. The highest density of CRH-containing neurones is found in the prefrontal, insular and cingulate areas. The distribution of CRH in these areas may explain its effects on cognitive processing (Palkovits & Brownstein, 1985).

Several groups of CRH neurones are also located in the brainstem and in the spinal cord (Palkovits & Brownstein, 1985). In the midbrain, CRH cells are found in the Edinger-Westphal nucleus, the periaqueductal grey and the dorsal raphe nucleus (Palkovits & Brownstein, 1985). In the pons, CRH cell bodies in the parabrachial nucleus project to the medial preoptic nucleus (Mpo) of the hypothalamus (Owens & Nemeroff, 1991). The locus coeruleus noradrenaline system also contains CRH neurones that may mediate the cross-talk between the two systems (Berridge & Waterhouse, 2003). In the medulla CRH neurones are known to exist in the NTS in the dorsal vagal complex, in addition to some scattered groups in the reticular formation and the spinal trigeminal nucleus (Palkovits & Brownstein, 1985). CRH cell bodies are found in laminae V, VI, VII and X of the spinal cord, as well as in the intermediolateral column of the thoracic and lumbar regions (Yao *et al,* 2004). Spinal CRH neurones have an important role in modulating sensory input via ascending projections to the thalamus and brainstem reticular formation (Chrousos, 1998).

An additional CRH-like neuropeptide urocortin has also been identified in the brain (Vaughan *et al,* 1995). Urocortin is a potent anorexigenic peptide of 40 amino acids that induces fed-like motor activity when administered centrally or peripherally in fasted animals (Kihara *et al,* 2001). Urocortin activates CRH receptor 1 and CRH receptor 2 (Torpy *et al,* 1999). I.c.v. administration of urocortin can also activate the HPA axis under basal conditions (Jamieson *et al,* 2006). Urocortin II and III also belong to the CRH family. Urocortin II is a 38 amino acid peptide which is highly selective for the CRH receptor two; it has been shown to have anorexigenic effects and hypotensive effects but it does not stimulate secretion of ACTH (Arai & Shibasaki, 2006). Urocotin III is also a 38 amino acid and similar to urocortin II it is

also highly selective for the CRH receptor two with anorexigenic effects but it does not stimulate ACTH secretion (Arai & Shibasaki, 2006).

1.6.2 Stress activates CRH release

Stress activates release of CRH from the hypothalamus and other areas within the brain (Habib *et al,* 2000). CRH initiates all aspects of the stress response including behavioural, autonomic and neuroendocrine responses (Behan *et al,* 1996). Stressors activate CRH neurones in the pPVN to stimulate CRH release into the hypothalamo-portal system; CRH neurones respond to acute and chronic stress in different ways. Acute stress increases CRH mRNA expression in the PVN after 2 hours (Harbuz *et al,* 1991; Imaki *et al,* 1991) with peak expression occurring 4 hours after stress (Ma *et al,* 1997). Intraperitoneal injection of hypertonic saline has been shown to increase CRH mRNA expression in the PVN (Harbuz *et al,* 1989). Parvocellular CRH heteronuclear (hn) RNA was also increased by administration of i.p. hypertonic saline (Ma & Aguilera, 1999). As discussed above, CRH is the dominant agent in the HPA axis response to acute stress, while AVP is more important in mediating responses to chronic or repeated stress (Harbuz & Lightman, 1992). Chronic osmotic stimulation decreases CRH mRNA expression in the PVN (Aguilera *et al,* 1994).

1.6.3. The CRH receptors

Receptor binding sites for CRH are found in the pituitary, brain and in various peripheral sites including the adrenal medulla, prostate, gut, spleen, liver, kidney and testes (Slominski *et al,* 2004). The CRH receptor is part of the seven-transmembrane G-protein-coupled family of receptors. CRH binding to its receptor increases

intracellular cAMP (De Souza & Battlaglia, 1988). The CRH receptors belong to the same family as the growth hormone releasing factor, parathyroid hormone (PTH), glucagon, vasoactive intestinal peptide (VIP), calcitonin and 5HT receptors (Parham *et al,* 2004).

Two distinct CRH receptor subtypes have been characterised; CRH-receptor 1 (CRH-R1) and CRH-receptor 2 (CRH-R2) (Beglinger & Degen, 2002). After cloning, expression of the CRH-R1 receptor was found in various parts of the brain with CRH binding sites found within the hypothalamus (De Souza *et al,* 1984). CRH-R1 expression is found in the rat forebrain, subcortical limbic structures in the septal region, amygdala, cerebral cortex and deep nuclei (Campbell et al, 2003). CRH-R1 expression was also found in the PVN, SON, ARC and SCN (Campbell et al, 2003), with high expression seen in the anterior and intermediate lobes of the pituitary (Potter *et al,* 1994).

The CRH receptor 2 (CRH-R2) was cloned (Lovenberg *et al,* 1995) and found to have two receptor splice variants; CRH-R2α and CRH-R2β. Expression of CRH-R2 is mainly found within the brain in subcortical structures with high expression found in the lateral septal nucleus, the VMH and in the choroid plexus. Very low expression of CRH-R2 has been seen in the pituitary (Chalmers *et al,* 1995).

1.6.4 Mechanisms by which stress turns on CRH brain systems

How stress turns "on" and "off" the CRH brain system is complex. Many neurotransmitters are involved in controlling and regulating CRH secretion. GABA inhibits CRH neurones (Givalois *et al,* 1998), whereas 5HT and cholinergic neurones stimulate CRH release (Itoi *et al,* 2004).

Noradrenaline and opioid peptides have both a stimulatory and inhibitory role on CRH release depending on the dose administered and the opioid/cholinergic receptor subtype involved (Jessop, 1999). Glucocorticoids potently inhibit CRH release by acting directly on the PVN; but direct corticosterone infused i.c.v. stimulates ACTH secretion (Laugero *et al,* 2001) this occurs through receptors in the hippocampus (Laugero *et al,* 2001). Glucocorticoids can also stimulate CRH neurones in the amygdala and the locus coeruleus noradrenaline system; this stimulatory affect may play a role in prolonging the effects of severe stress by creating a positive feedback loop between CRH and noradrenaline systems (Spinedi *et al,* 1988).

1.6.5. Expression of CRH receptors following stress

Normally, there is a balance between the availability of CRH peptide and the number of available CRH receptors. Stress results in hypersecretion of CRH and a decrease in the number of available CRH receptors in the anterior pituitary (Herman *et al,* 1995). Chronic administration of corticosterone causes a decrease in the number of CRH receptors in the anterior pituitary (Hauger *et al,* 1988) as does i.p. hypertonic saline (Aguilera *et al,* 2001). Lesion of the PVN that reduces CRH secretion increases the number of CRH receptors in the pituitary (De Souza *et al,* 1987). Expression of CRH-R1 mRNA in the PVN is substantially increased by stress (Imaki *et al,* 1996). Both restraint stress and i.c.v. administration of CRH increase CRH-R1 mRNA expression in the PVN (Imaki *et al,* 1996), as does immune challenge and i.p. hypertonic saline (Mansi *et al,* 1996; Rivest *et al,* 1995). Increases in CRH-R1 mRNA expression can be seen in the PVN 1-2 hours after increases in ACTH and

corticosterone secretion (Imaki *et al,* 1996). These findings indicate that there is local feedback control of CRH neurone activity by CRH.

1.7. Arginine vasopressin (AVP)

AVP is released from the posterior pituitary when the body is dehydrated. It is produced in the hypothalamus, and also has various functions in the brain. Most of the AVP in the body is stored in the posterior pituitary to be released into the bloodstream; however some of it is released directly into the brain (Ring, 2005). AVP is secreted from the posterior pituitary gland in response to changes in plasma volume and osmotic pressure. The neurones that make AVP in the SON and PVN are also osmoreceptors (Bourque & Oliet, 1997).

Another important source of AVP is that released from the pPVN into the hypothalamo-pituitary portal system whence it stimulates release of ACTH (Da Costa *et al,* 1996).

1.7.1 The role of AVP in the stress response

AVP neurones in the pPVN in the hypothalamus have an important role in the stress response. AVP works together with CRH during stress to stimulate the secretion of ACTH (Koob *et al,* 1985). A subset of pPVN neurones synthesise and secrete CRH and AVP, whereas another subset secretes CRH or AVP only (Charmandri *et al,* 2005). The subset that secretes both neuropeptides enlarges significantly during stress (Hauger *et al,* 1993). pPVN neurones expressing AVP project to noradrenergic neurones of the brainstem and the hypophyseal portal system in the median eminence (Buckingham, 2006).

AVP neurones also project to POMC-containing neurones in the ARC which in turn project to the PVN CRH and AVP neurones (Herman *et al,* 1997). During chronic or prolonged stress, there may be a shift in the control of ACTH from CRH to AVP (Hauger & Aguilera, 1993).

1.7.2. Stress activates AVP release

AVP controls ACTH secretion mainly by regulating the effect of CRH in the pituitary corticotroph (Gillies & Lowry, 1979). In control non-stressed rats, approximately 50% of CRH-containing neurones in the pPVN co-express AVP. Acute stress increases AVP hnRNA expression in the pPVN 1-2 hours after the onset of stress (Ma *et al,* 1997) and AVP mRNA expression 4 hours after the onset of stress (Ma *et al,* 1997). Chronic stress enhances activity of the pPVN AVP system (Harbuz & Lightman, 1992). Levels of CRH hnRNA and mRNA, which increase following acute stress, become progressively less if rats have been repeatedly restrained for 14 days (Ma *et al,* 1997). The levels of AVP mRNA in the pPVN progressively increase however in response to repeated restraint stress (Bartanusz *et al,* 1993). These data suggest that AVP synthesised in the pPVN plays a more important role in maintaining HPA axis activity under conditions of chronic stress (Ma & Lightman, 1998).

1.7.3. Glucocorticoid feedback

Glucocorticoids restore basal AVP mRNA expression following a stress response (Herman, 1995; Kiss *et al*, 1984). Glucocorticoids inhibit transcription of AVP by

binding to the glucocorticoid regulatory element in the vasopressin promoter region (Pearce *et al,* 1988; Uht *et al,* 1988).

1.7.4. Control of AVP synthesis

AVP hnRNA expression does not peak until 1 hour after acute stress, unlike CRH hnRNA which peaks 5 minutes after (Imaki *et al,* 1995). The promoter region of the CRH gene does not contain the AP-1 sequence to which many transcription factors bind (Seasholtz *et al,* 1988) unlike the AVP gene which does possess an AP-1 binding site (Pardy *et al,* 1992). Transcription factors such as Fos and NGFI-B binding to the AP-1 site have been implicated in inducing AVP gene responses to stress (Che *et al,* 1993).

1.7.5. AVP receptors

AVP stimulates ACTH secretion by binding to the $V1_b$ receptor in the pituitary (Lolait *et al,* 1995). AVP receptors, of which there are three types, belong to the G-protein linked super family. The V_{1a} receptor has been found in the liver, vascular smooth muscle, platelets (Laszlo *et al,* 1991), brain (Tribollet *et al,* 1988), and mesangial cells (Jard *et al,* 1987). AVP binding to the V_{1a} receptor activates phospholipase C, which stimulates phosphatidylinositol production to increase intracellular calcium and stimulate protein kinase C (Laszlo *et al,* 1991). AVP binding to the V_{1b} receptor also stimulates phosphatidylinositol production, increasing intracellular calcium like V_{1a} activation (Tanoue *et al,* 2004). The V_{1b} receptor is found mainly in the anterior pituitary where it mediates AVP stimulation of ACTH release. The V_2 receptor is the final member of the family and works by

activating the heterotrimeric G protein G_s, promoting stimulation of adenylate cyclase (Birnbaumer *et al,* 1990). The V_2 receptor is expressed mainly in the cells of the renal collecting ducts where it plays a key role in water homeostasis (Carmosino *et al,* 2006).

1.7.6. Regulation of V_{1b} receptor

V_{1b} receptor expression in the anterior pituitary is increased during chronic stress (Aguilera & Diehl, 2000). Removal of increased glucocorticoids by adrenalectomy (ADX) decreases $V1_b$ receptor mRNA expression (Aguilera & Rabadhan-Diehl, 2000). Administration of gluocorticoids decreases pituitary AVP binding but increases V_{1b} receptor mRNA (Antoni et al, 1995). This change where pituitary binding is decreased and V_{1b} receptor mRNA increased suggests that V_{1b} receptor levels depend on post-transcriptional mechanisms (Aguilera et al, 2003). It is thought the interaction between glucocorticoids and vasopressin play an important role in regulating anterior pituitary V_{1b} receptor mRNA expression during alterations of the HPA axis.

1.8. Oxytocin

Oxytocin is structurally very similar to vasopressin; it is made in the SON and the magnocellular PVN and released from the posterior pituitary into the blood. Oxytocin is also made by neurones within the PVN that project to other parts of the brain and spinal cord (Ranson *et al,* 1998). Stress also increases oxytocin release into the blood, increasing oxytocin levels 2-3 fold (Lang *et al,* 1983; Gibbs, 1984). Oxytocin secretion increases alongside ACTH secretion although some stressors do

not increase peripheral oxytocin secretion (Engelmann *et al,* 1999). Oxytocin can also act centrally, but once secreted from the pituitary it cannot re-enter the brain (Rinaman *et al,* 2000). Oxytocin that controls the responses of the HPA axis is thought to more likely come from the magnocellular neurones within the PVN, but may be secreted in the median eminence into the capillaries of the portal system from axons of both PVN and SON neurons (Wotjak *et al,* 2001). Also, oxytocin is released either by dendrites or cell bodies within the magnoceullular PVN or SON where it can act locally on nearby neurones and synaptic contacts (Ludwig & Leng, 2006). Oxytocin has also been described as an anti-stress factor within the brain, as central administration of an oxytocin antagonist increases basal and stress-induced ACTH secretion (Neumann *et al,* 2000). Also daily peripheral injections of oxytocin over a five day period also decrease blood pressure (Moberg *et al,* 1988). Oxytocin receptors have been found to be expressed in the brain and spinal cord including the amygdala, VMH and brainstem (Tribollet *et al,* 1991). Oxytocin may also act in other brain regions that express the oxytocin receptor to play a role in feeding (Sabatier, 2006) and in reproductive behaviours (Pedersen & Boccia, 2006).

1.9. Circadian regulation of HPA activity

Connections between the PVN and suprachiasmatic nucleus (SCN) control the circadian rhythm of glucocorticoid secretion through regulation of pPVN CRH neurons. Connections can be both direct and indirect. Lesions of the SCN stop the rhythmical control of cortisol secretion (Moore & Eichler, 1972). In humans the lowest peak in cortisol secretion occurs about midnight. Cortisol levels rise about 2-3 hours after sleep onset (Van Cauter *et al*, 1994) and continue to rise until morning.

The peak in cortisol occurs at about 9am.Cortisol levels decline throughout the day. Rats are nocturnal animals and their plasma levels of ACTH and corticosterone peak in the evening and are lowest in the morning (Dallman *et al*, 1978). CRH is also released in a circadian-dependent pulsatile fashion from the parvocellular cells of the PVN (Hauger & Datzenberg, 2000), although CRH and cortisol levels in the aCSF are not under circadian rhythm (Kling *et al,* 1991). CRH gene transcription is however under circadian control; CRH gene transcription in the PVN increases during the night and decreases in the early morning in humans (Watts *et al,* 2004).

1.10. The relationship between glucocorticoids and energy balance

After acute stress the increased adrenaline and sympathetic neural activation characteristic of a stress response interact to increase blood glucose to ensure the brain has adequate amounts. As discussed above glucocorticoids inhibit activity of the HPA axis through feedback mechanisms (Di *et al,* 2003). Increased corticosterone concentration also leads to increases in food ingestion, especially sweet and fatty food (Ipel *et al,* 2001). It is known that there are very close relationships between feeding, metabolism and energy storage and glucocorticoid secretion. In the presence of low insulin level, glucocorticoid concentrations are elevated. Glucocorticoids are a key requirement when energy stores are low, and they do not serve to inhibit the HPA axis in this state (Dallman *et al*, 2004). Adrenalectomised (ADX) rats, with low glucocorticoid level, increase their caloric intake with high energy diets; the increased caloric intake alters CRH expression in the PVN and the amygdala and none of the normal effects characteristic of ADX are seen (Dallman *et al,* 2004).

An extensive neural system that regulates energy balance and controls activity of the HPA axis has begun to emerge (Dallman *et al,* 1995). It is known that glucocorticoids stimulate feeding and insulin secretion (Dallman *et al,* 1995), but also mobilise energy stores peripherally, yet insulin inhibits food intake (Dallman *et al,* 1995). Centrally, insulin inhibits NPY mRNA expression but corticosteroids stimulate it (Dallman *et al,* 2005). It seems that there may be reciprocal actions of insulin and corticosterone at the level of the NPY neurones controlling food intake.

1.10.1. Appetite control – hypothalamic neuropeptides

The hypothalamus has been known to play a major role in energy regulation for a long time. The VMH has often been referred to as the "satiety" centre and the LHA the "hunger centre" (Stellar, 1997). Rather than these nuclei specifically controlling hunger and satiety it is thought that neuropeptides signalling via interconnected neuronal circuits are in fact regulating energy balance (Schwartz *et al,* 2003). The ARC is known to play a key role in regulating appetite. There are two major groups of neurones in the ARC which control energy homeostasis (Cone *et al,* 2001), the first inhibits food intake via expression of POMC and cocaine-amphetamine-regulated-transcript (CART) (Elias *et al,* 1998), the second stimulates food intake via expression of NPY and agouti-related-peptide (AgRP) (Broberger *et al,* 1998).

NPY

High levels of NPY are seen in the CNS (Allen et al, 1983). NPY mRNA expression increases with fasting and when administered centrally NPY stimulates release of insulin and reduces energy expenditure independently of increased food intake (Moltz & McDonald, 1985). NPY belongs to the pancreatic polypeptide family; it

binds to G-protein coupled receptors called Y1-Y6 (Larhammar, 1996). The orexigenic effects of NPY are thought to be mediated through the Y1, 2, 4 and 5 receptors (Fekete *et al,* 2002).

POMC and CART

POMC mRNA expression in the ARC decreases with fasting (Schwartz *et al,* 1997). The melanocortins are produced from POMC and bind to the melanocortin receptor, both melanocortin 3 and melanocortin 4 receptors are expressed in the ARC, VMH and PVN and have been implicated in reducing food intake (Mountjoy *et al,* 1997). CART mRNA also decreases with fasting (Kristenson *et al,* 1998); it is coexpressed with α-MSH in the ARC and when administered centrally both CART and α-MSH decrease food intake (Aja *et al,* 2001).

1.10.2. Downstream pathways

Hypothalamic nuclei such as the PVN, DMH, LHA and perifornical area receive NPY/AgRP and POMC/CART neuronal projections from the ARC (Elias *et al,* 1998). These areas contain secondary neurones which process information regarding energy homeostasis. A number of signalling molecules that are expressed in these regions have been shown to be physiologically involved in energy homeostasis.

PVN

The PVN receives projections from the ARC and is sensitive to many neuropeptides implicated in energy homeostasis including NPY (Lambert *et al,* 1995), ghrelin (Lawrence *et al,* 2002), orexin (Edwards *et al,* 1999) and leptin (Van Dijk *et al,* 1996). Neurones projecting to the PVN which express NPY have been shown to

reduce GABAergic signalling unlike neurones projecting to the PVN that express POMC which increase GABAergic signalling (Cowley *et al,* 1994).

DMH and VMH

As well as indirect projections with other hypothalamic nuclei involved in energy homeostasis the DMH receives direct projections from the NPY neurones in the ARC (Kalra *et al,* 1999). The VMH has long since been thought to be the "satiety" centre in energy homeostasis (Stellar, 1997) and receives projections from both the NPY and POMC expressing neurones in the ARC (Stellar, 1997).

LHA

The LHA contains neurones that express melanocyte concentrating hormone (MCH). (Marsh *et al,* 2002). Centrally administered MCH decreases food intake in mammals (De Lecea *et al,* 1998). Orexin is also a peptide that is produced in the LHA although it is produced in neurones separate to those that produce MCH (De Lecea *et al,* 1998). Orexin neurons (A and B) project widely throughout the brain, including to the PVN and ARC (De Lecea *et al,* 1998). Centrally administered orexin-A stimulates feeding (Haynes *et al,* 1999) and peripheral administration increases insulin secretion (Novak *et al,* 2000), and the HPA axis (Taheri & Bloom, 2001).

1.10.3. Brainstem pathways

The brainstem plays a critical role in energy homeostasis, particularly via the nucleus tractus solitarius; this is in close proximity to the area postrema, just like the ARC which is in close proximity to the blood brain barrier-free zone in the median eminence, which means it can react to peripherally circulating peptides (Ellacott &

Cone, 2004). Y1 and Y5 receptor expression have been identified within the NTS (Dumont *et al,* 1998).

1.10.4. Reward pathways

Reward circuitry regulating food intake is complicated (Wang et al, 2006). Levels of insulin and leptin within the body switch "on" and "off" reward pathways, involving the mesolimbic circuit (Levin, 2006). Other systems are also involved including opioids where increased opioid can reduce the volume of food desired without actually reducing hunger (Bodnar et al 2005; Yeomans *et al,* 1990). Dopamine has also been implicated in this process. Centrally administered opioid and dopamine antagonists cause an increase in the ingestion of sweet and fatty foods (Zhang & Kelley, 2000). As discussed above the signals that activate the reward pathways are peptides from the peripheral circulation.

1.10.5. Peripheral signals of adiposity

Leptin

Leptin is a peptide secreted from adipose tissue that plays a key role in energy homeostasis. It is transported across the blood brain barrier into the ARC (Banks *et al,* 1996) where it binds to its receptor, a member of the cytokine family (Tartague *et al,* 1995). Leptin receptor expression has been seen in various hypothalamic areas including the ARC, VMH, DMH and LHA (Fei *et al,* 1997). Greatest expression is seen in the ARC, specifically in the NPY neurones (Mercer *et al,* 1996) and the POMC neurones (Stephens *et al*, 1995).

Insulin

Insulin is produced in the pancreas (Schwartz *et al,* 1992) and circulating levels increase rapidly after a meal (Polonsky *et al,* 1988). Like leptin, insulin crosses the blood brain barrier through a saturable process (Baura *et al,* 1993). On entering the brain it reduces food intake (Ikeda *et al,* 1986), however studies investigating the effects of insulin on food intake are complicated by the fact that hypoglycaemia caused by increasing levels of insulin increases food intake (Friedman & Granneman, 1983). Insulin binds to its receptor which has tyrosine kinase activity and is composed of two sub-units (Espinosa-Paez *et al,* 1998); the receptor is distributed widely in the brain, particularly in the hypothalamus including the ARC, DMH and PVN (Corp *et al,* 1986) and also peripherally in muscle and liver.
As well as the peripheral signals insulin and leptin, other peripheral signals from the GI tract are also important in regulating energy homeostasis.

1.10.6. Peripheral signals from the GI tract

Ghrelin

Ghrelin is a 28 amino acid peptide released from the stomach but also from the testis, placenta, kidney, pituitary, intestine, evidently synthesised in a group of neurones next to the third ventricle which project to the NPY, POMC and CRH neurones (Cowley *et al,* 2003). Ghrelin neurones in the brain also project to the orexin neurones in the LHA (Toshinai *et al,* 2003) and i.c.v. administration of ghrelin stimulates orexin neurones (Lawrence *et al,* 2002). Circulating ghrelin levels fall just after a meal (Tschop *et al,* 2000). Ghrelin acts on the growth hormone secretagogue receptor (GHS-R) and it stimulates growth hormone (GH) release either from the

pituitary or by acting on the GHS-R in the brain, through which GH secretion is stimulated (Kojima *et al,* 1999). GHS-R's are found widely including the hypothalamus, pituitary, stomach, intestine, pancreas, liver, kidney and placenta (Date *et al,* 2000). Ghrelin stimulates food intake following peripheral administration in humans; in rats it increases Fos expression in NPY containing neurones in the ARC (Toshinai *et al,* 2003).

1.11 Appetite-regulating neuropeptides and the HPA axis

The relationship between the HPA axis and factors relating to adipose tissue storage are very important in the context of stress and obesity (Dallman *et al,* 1995). Many neuropeptides that have been implicated in the control of appetite have also been shown to activate the HPA axis. These include insulin, ghrelin, orexin and neuropeptide Y (NPY).

1.11.1 Insulin Induced Hypoglycaemia (IIH)

Insulin

Insulin is secreted peripherally from the pancreas, and it can enter the brain. In the ARC it inhibits the NPY/AgRP neurones and activates the POMC/CART neurones, reducing appetite (Porte et al, 2002) (Fig.1.2). Insulin induced hypoglycaemia (IIH) has been shown to activate the HPA axis (Plotsky et al, 1985; Jezova et al, 1987). Peripheral administration of insulin stimulates release of ACTH, β-endorphin, GH and prolactin (PRL) (Jezova *et al,* 1987). While insulin reduces food intake, hypoglycaemia caused by insulin injection increases food intake (Friedman & Granneman, 1983). Similarly studies investigating the effect of IIH on the HPA axis

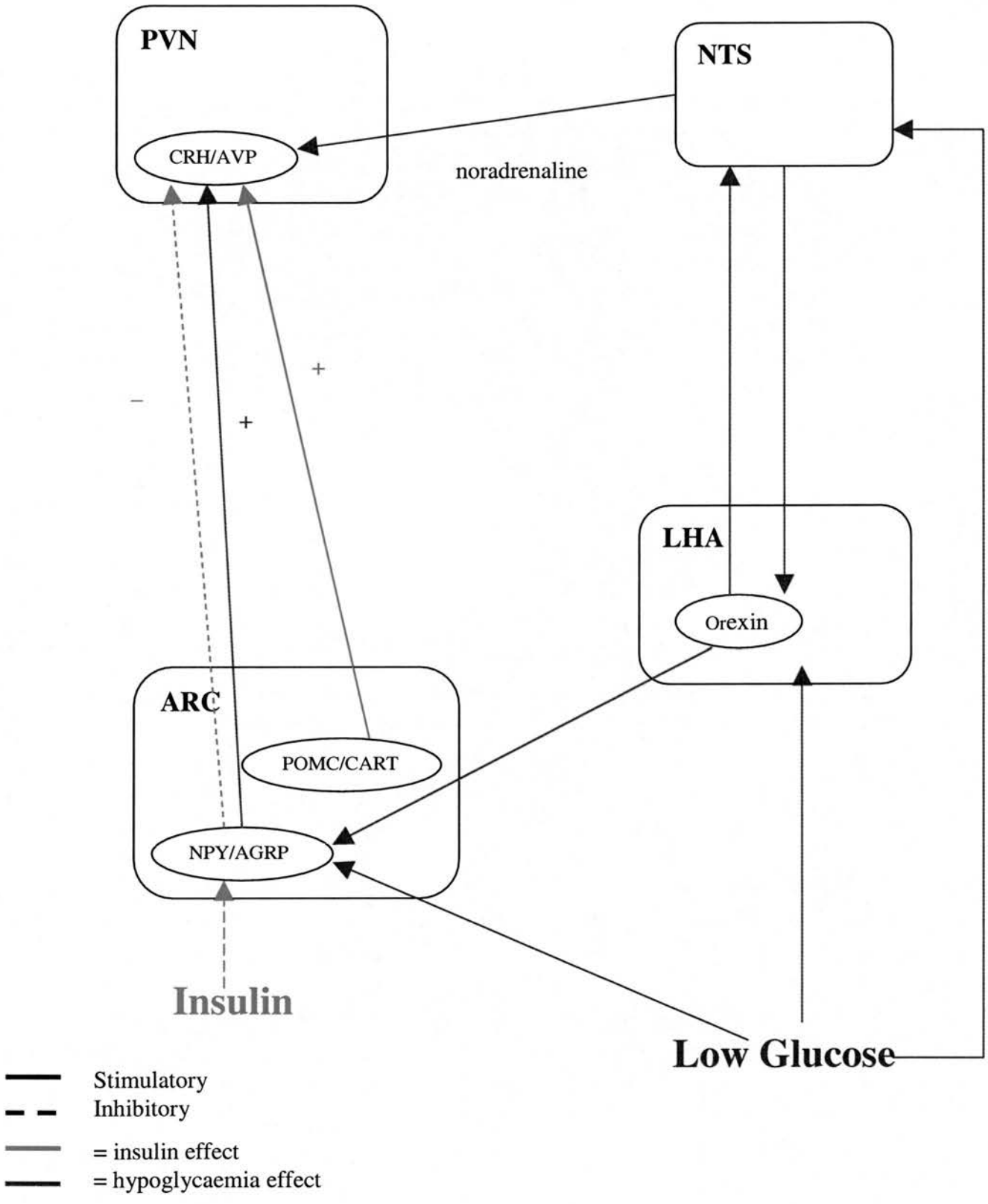


Figure 1.2: Central processing of insulin induced hypoglycaemia and the HPA axis PVN = paraventricular nucleus; CRH = corticotrophin releasing hormone; AVP = arginine vasopressin; NTS = nucleus solitary tract; LHA = lateral hypothalamic area; ARC = arcuate nucleus; NPY = neuropeptide Y; AgRP = agouti related peptide; POMC = pro-opiomelanocortin; CART = cocaine and amphetamine regulated transcript

are complicated by the contrasting effects of insulin and hypoglycaemia on neurones within the brain.

Hypoglycaemia

The LHA is importantly involved in controlling responses to IIH. It contains glucose sensitive neurones that are stimulated by hypoglycaemia. It is thought that the glucose sensitive neurones are activated mainly indirectly from projections in the brainstem which receive information from glucoreceptors in the gut and liver (Paton *et al,* 2000). Hypoglycaemia has been shown to increase Fos expression in the NTS (Cai *et al,* 2001). The glucose sensitive neurone accounts for around 25% of the LHA neurones, some of these have also been shown to express the orexin peptides (Moriguchi *et al,* 1999). The orexins have been importantly implicated in the control of food intake. Pre-pro orexin mRNA levels have been shown to be increased by IIH but only when food was not available (Sakurai *et al,* 1998). When food was freely available the increase in orexin mRNA seen by falling blood glucose was not seen anymore (Cai *et al,* 1999). The orexin neurones may be very important then in the control of short-term feeding behaviour, in particular in response to reduced blood glucose level.

1.11.2 Orexin activates the HPA axis

The orexins have been shown to activate the HPA axis directly and indirectly (Bornstein *et al,* 1997). Central administration of orexin-A has been shown to increase Fos expression in the pPVN (Kuru *et al,* 2000) and also CRH mRNA levels in the pPVN (Brunton *et al,* 2003). Orexin activates neurones in the PVN indirectly via the NPY neurones in the ARC because the stimulatory effect of orexin-A on

CRH release in hypothalamic explants were blocked by a Y1 receptor antagonist (Russell *et al,* 2001), and administration of an NPY antagonist *in vivo* blocked the increase in plasma corticosterone normally seen (Jaszberenyi et al, 2001).

As well as mediating the effects of orexin on the HPA axis, NPY administration also activates the HPA axis (Horvath *et al,* 1999).

1.11.3 NPY activates the HPA axis

Orexigenic NPY neurones are located in the ARC and the NTS (Heilig & Widerlov, 1993) and project directly to the PVN (Liposits *et al,* 1998). NPY has been shown to stimulate CRH release and CRH mRNA expression (Suda et al, 1993). The HPA axis can be stimulated by NPY given via various routes. The greatest CRH release was seen however when NPY was injected directly into the PVN (Haas *et al,* 1989).

1.11.4 Ghrelin activates the HPA axis

Central administration of the neuropeptide ghrelin increases Fos expression in NPY containing neurones in the ARC, and activates the HPA axis; ghrelin does not activate the HPA axis when given systemically (Wren *et al,* 2000). It also stimulates GH secretion when administered centrally suggesting a hypothalamic site of action (Kojima *et al,* 1999). Ghrelin acts on the HPA axis by activating NPY neurones and stimulating the pPVN CRH neurones (Wren *et al,* 2000) (Fig.1.3). Ghrelin stimulates release of both CRH and NPY from hypothalamic explants (Wren *et al,* 2000).

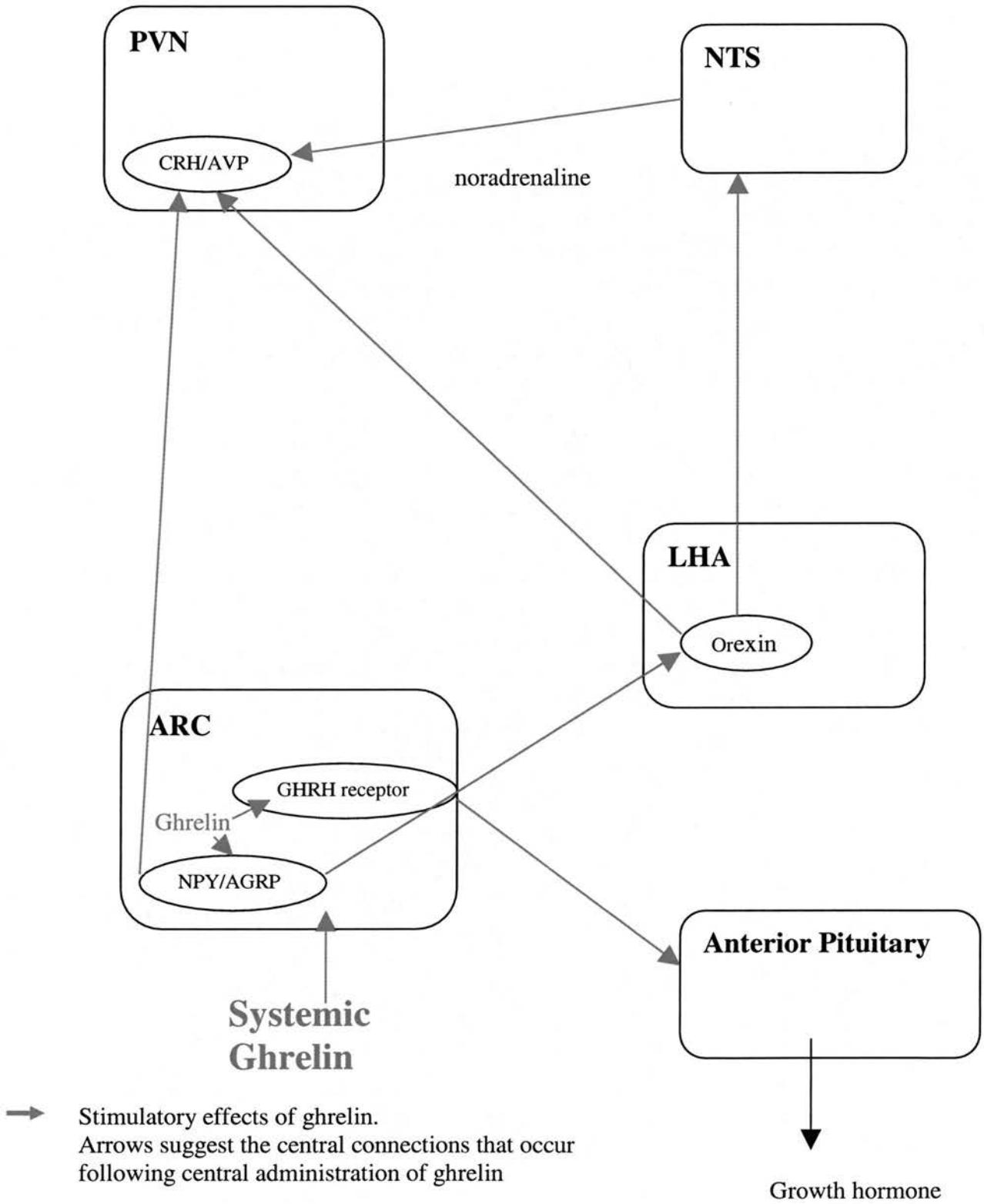


Figure 1.3: Central processing of Ghrelin actions and the HPA axis
PVN = paraventricular nucleus; NTS = nucleus of the solitary tract; LHA = lateral hypothalamic area; ARC = arcuate nucleus; NPY = neuropeptide Y; AgRP = agouti related peptide; GHRH = growth hormone releasing hormone; CRH = corticotrophin releasing hormone; AVP = arginine vasopressin

1.12 Neuroendocrine adaptations to stress in pregnancy

As discussed, individuals can experience either internal or external stressors. Internal stressors can be physical or psychological. Stressors can also be defined as those that arise suddenly over a short period of time, acute stressors; or those that develop over a longer period of time, chronic stressors (Kennedy *et al,* 1998). In chronically stressed animals the response of the HPA axis to a stressor is reduced (Gomez *et al,* 1996). Pregnancy also acts in a similar way to a chronically stressed state where the response of the HPA axis to a stressor is reduced during late pregnancy (Neumann *et al,* 1998). As well as reduced responses of the HPA axis to stress, the basal activity of the HPA axis also changes during late pregnancy (Johnstone *et al,* 2000; Atkinson & Waddell, 1995).

1.12.1 Basal activity of the HPA axis during pregnancy

PVN: CRH and AVP expression

Basal CRH mRNA expression in the pPVN is reduced during late pregnancy. There is no change in the ratio of AVP to CRH mRNA indicating that basal AVP expression is reduced also (Johnstone et al, 2000). Reduced basal expression could be due to reduced forward drive or increased glucocorticoid feedback (Johnstone *et al,* 2000).

ACTH and corticosterone

Morning basal levels of ACTH in rats have been reported not to change through pregnancy (Neumann *et al,* 1998). However, the circadian increase in ACTH secretion normally seen in the evenings is reduced in late pregnant rats (Atkinson &

Waddell, 1994). Basal levels of corticosterone change little throughout pregnancy, except for an increase seen on day 22 of pregnancy (Neumann *et al,* 1998; Atkinson & Waddell, 1994), with reduced plasma corticosterone levels during early pregnancy (Ogle & Kitay, 1977; Atkinson & Waddell, 1994).

1.12.2. Responses of the HPA axis to stressors in pregnancy

The HPA axis is hyporesponsive to both emotional and physical stressors during late pregnancy (Neumann *et al,* 1998). Specifically ACTH, corticosterone and oxytocin secretory responses to forced-swimming are reduced in rats (Neumann *et al,* 1998) and in mice (Douglas *et al,* 2003). HPA axis responses to immune challenge with systemic endotoxin or IL-1β are also reduced during late pregnancy (Brunton *et al,* 2005). I.c.v. orexin-A activates the HPA axis in virgin female rats but does not increase plasma ACTH, corticosterone or CRH mRNA expression in the pPVN in late pregnant rats (Brunton *et al,* 2003). Reduced responsiveness during late pregnancy could be due to several reasons, including changes in limbic feedback systems (Johnstone *et al,* 1997), changes at the corticotrophs of the anterior pituitary (Neumann *et al,* 1998) or within the CRH/AVP neurones in the hypothalamus or their inputs (Douglas & Russell, 2004).

1.12.3 Changes in limbic feedback systems

Changes in feed-forward activation of the HPA axis are found during late pregnancy, including reduced responses to stress in regions of the limbic system that project to the PVN (de Costa *et al,* 1996). The brain noradrenergic system is also restrained in late pregnancy (Douglas *et al,* 2005) and there is reduced release of noradrenaline

within the PVN after systemic IL-1β as well as reduced α_{1A}-receptor mRNA expression in the PVN (Brunton *et al,* 2005; Douglas *et al,* 2005). Reduced noradrenergic release or action looks as if it is likely to contribute to reduced activation of the HPA axis responses to stress; this has been shown to be partly due to opioid inhibition (Brunton *et al,* 2005).

Enhanced glucocorticoid feedback could also be an explanation for reduced ACTH secretion. Evidence of altered feedback was sought by looking at expression of the mineralocorticoid (MR) and gluocorticoid (GR) receptor during late pregnancy. MR and GR expression did not change in pregnant rats, except in the dentate gyrus where GR mRNA expression was increased (Johnstone *et al,* 2000). 11β-HSD1 activity has been shown to be increased 3-fold in the anterior pituitary by day 21 of pregnancy (Johnstone *et al,* 2000). 11β-HSD1 converts corticosterone from its inert form, so that increased 11β-HSD1 activity in the anterior pituitary in pregnancy is expected to increase glucocorticoid levels at this site, thus increasing negative feedback by corticosterone (Johnstone *et al,* 2000). Administration of an 11β-HSD1 inhibitor, glycyrrhetinic acid (GA), did not however increase the ACTH secretory response to forced swimming (Johnstone *et al,* 2000). In contrast, pharmacological adrenalectomy increased ACTH secretion similarly in late pregnant and virgin rats (Johnstone *et al,* 2000). It may be that pregnant rats are less sensitive to rapid glucocorticoid feedback but do not change sensitivity to delayed feedback (Johnstone *et al,* 2000); this suggests reduced neural drive to the PVN may account for hyporesponsiveness of the HPA axis to acute stress during late pregnancy.

1.12.4 Anterior pituitary mechanisms in reduced HPA responsiveness during late pregnancy

Another reason for reduced responsiveness of the HPA axis during late pregnancy may be changes in the corticotrophs of the anterior pituitary. The number of CRH receptors in the anterior pituitary is reduced on day 11 of pregnancy, although a reduction in CRH-stimulated cAMP production was not seen until day 17 of pregnancy (Neumann *et al,* 1998). The finding of a reduced number of CRH receptors without changes in cAMP production indicates that during late pregnancy reduced ACTH secretory responses are likely a result of changes at higher levels of the HPA axis (Neumann *et al,* 1998). Changes in receptor numbers in the anterior pituitary does not lead to reduced ACTH responses when CRH and AVP are both given together (Ma *et al,* 2005). There is evidence that reduced release of AVP rather than CRH accounts for reduced ACTH secretion in response to certain stressors during late pregnancy (Ma *et al,* 2005).

1.12.5 Hypothalamic mechanisms of reduced responsiveness during late pregnancy

It is possible that reduced neural drive to the PVN CRH/AVP neurones may account for the hyporesponsiveness of the HPA axis rather than enhanced negative feedback or changes in the anterior pituitary. *C-fos* mRNA expression in the pPVN is significantly less after restraint stress than in virgin rats (da Costa *et al,* 1996). Orexin-A exerts its effects on the HPA axis by acting centrally at the level of the pPVN CRH/AVP neurones. The HPA axis has been shown to be hyporesponsive to centrally administered orexin-A during late pregnancy (Brunton *et al,* 2003). This

indicates that the CRH/AVP neurones are less responsive to excitatory stimuli which could be a consequence of either reduced neural drive or enhanced inhibitory inputs to the CRH neurones (Brunton *et al,* 2003).

1.12.6 Oxytocin secretory responses to stress during late pregnancy

During late pregnancy as well as reduced ACTH and corticosterone responses, oxytocin secretory responses to stressors are also reduced. The content of oxytocin in the posterior pituitary increases 3-fold at the end of pregnancy; this is due to decreased secretion, conserving stores of oxytocin for when they are needed during parturition (Russell *et al,* 2003). Possible reasons for reduced oxytocin responses include a reduced ability of the oxytocin system to respond due to either reduced effectiveness of excitatory input or increased effectiveness of inhibitory inputs. Picrotoxin, a $GABA_A$ receptor antagonist (which interrupts tonic inhibition of oxytocin neurones), stimulates oxytocin secretion in both virgin and pregnant rats (Soafe *et al,* 2004).

Inhibition by endogenous opioids and neuroactive steroid metabolites of progesterone are also thought to account for reduced oxytocin secretory responses to stress during late pregnancy.

1.12.7 Endogenous opioids in pregnancy

Endogenous opioids restrain stimulation of oxytocin neurones during late pregnancy (Douglas *et al,* 1995). Oxytocin secretion in response to IL-1β was enhanced by naloxone (the opioid receptor antagonist) indicating endogenous opioids may mask an exaggerated response (Brunton *et al,* 2006).

Secretion of prolactin (another stress hormone) has also been shown to be inhibited during late pregnancy by endogenous opioids (Soaje & Deis, 1994). Naloxone also restored the suppressed ACTH and corticosterone responses normally seen during late pregnancy (Douglas *et al,* 1998). It is thought that naloxone may be exerting its effects on the HPA axis via the hypothalamus (Wang *et al,* 1996), likely through the CRH neurones. Hyporesponsiveness of the HPA axis to immune challenge during late pregnancy is a result of endogenous opioid inhibition of the pPVN CRH neurones (Brunton *et al,* 2005). Endogenous opioids may act by suppressing noradrenaline release from the PVN. Naloxone triggered a pPVN CRH mRNA response to IL-1β and a noradrenaline response when microdialysed directly into the PVN (Brunton *et al,* 2005). Naloxone does not distinguish between opioid receptor subtypes; although the hypothalamus is almost devoid of δ receptors (Mansour *et al,* 1987). Increased μ receptor mRNA and pro-enkephalin A mRNA expression have been seen in the NTS of pregnant rats (Brunton *et al,* 2005). Increased opioid receptor and peptide production in the NTS could lead to increase transport to terminals in the PVN; μ opioids inhibit noradrenaline release in the SON (Onaka *et al,* 1995) and the PVN (Brunton *et al,* 2005).

1.12.8 Sex Steroids during late pregnancy

Neuroactive steroid metabolites of progesterone are also involved in reduced HPA axis and oxytocin responses to stress in late pregnancy. The neuroactive metabolite allopregnanolone is produced in the brain, and enzymes converting progesterone to allopregnanolone are expressed in the medulla (Khanna *et al,* 1995). As pregnancy reaches term the amount of allopregnanolone produced decreases (Koncas *et al,*

1998). Late pregnant rats treated with finasteride (a 5α-reductase inhibitor; where 5α-reductase is an enzyme involved in converting progesterone to allopregnanolone) 20 and 2 hours before administration of IL-1β showed a significant oxytocin secretory response in pregnant rats with no response seen in pregnant rats given vehicle and IL-1β alone (Brunton *et al,* 2005). It is possible that allopregnanolone produced in the medulla may act on neurones in the NTS and participate in inducing the opioid inhibition seen at this time. As well as inducing opioid expression by acting on noradrenaline neurones in the medulla it is possible that allopregnanolone may act independently via the $GABA_A$ receptors on oxytocin and CRH neurones.

1.12.9. Placental CRH and POMC

CRH is synthesised and released by the human placenta (Goland *et al,* 1986) and levels of CRH mRNA increase in the last 5 weeks of pregnancy (Frim *et al,* 1988). CRH mRNA has also been shown in gorilla, monkey (Robinson *et al,* 1989) and sheep placenta (Jones *et al,* 1989) but it has not been seen in guinea pig or rat placenta (Robinson *et al,* 1989). Because CRH mRNA is not seen in the rat placenta, placental CRH is not involved in reduced responsiveness of the HPA axis in the rat. The pregnant rat is a good model therefore to look at the adaptations of the maternal HPA axis during late pregnancy.

1.12.10. CRH binding protein

CRH-binding protein (CRH-BP) may regulate CRH activity during pregnancy. CRH-BP is a 37 kDa glycoprotein produced by the liver and brain (Orth *et al,* 1987). In the brain CRH-BP is expressed predominantly in the cerebral cortex; it colocalises with

CRH and CRH receptors in several brain regions (Potter *et al,* 1992). This co-expression suggests that the CRH-BP may alter the interaction of CRH with the CRH receptor. In the brain 40-60% of CRH is bound to the CRH-BP (Behan *et al,* 1995). As well as been expressed in the brain and liver (Potter *et al,* 1991) it has also been shown to be expressed in the human placenta (Petraglia *et al,* 1993). It is possible that the CRH-BP can regulate peripheral CRH activity in women during pregnancy (Linton *et al,* 1990), and it is known that most of the CRH in the peripheral circulation during pregnancy is bound to a 37 kDa protein (Orth *et al,* 1987). This mechanism would not operate in pregnant rats, but a role for altered brain CRH-BP in pregnant rats has not been explored, but is another possible mechanism for reduced responses at this time.

1.13. Fetal programming

Exposure of women to exogenous agents including toxins, drugs and hormones during pregnancy can alter the development and organisation of specific tissues (Csaba, 1986). Males have been shown to have a burst of androgen secretion around the time of birth; the extra androgen controls the neurochemistry of specific hypothalamic nuclei (Arai *et al,* 1968). Oestrogens can also affect the developing CNS (Simerley *et al,* 2002). This increase in certain steroid hormones and their organisational actions are effective only at specific perinatal periods and determine gender specific control of reproductive processes in the brain, but their effects persist throughout life. Glucocorticoids during pregnancy can also have long term effects on the foetus.

1.13.1 Glucocorticoid programming

Glucocorticoids and birth weight

Glucocorticoid administration during pregnancy reduces birth weight in animal models (Reinisch *et al,* 1978) and humans (French *et al,* 1998). Greatest reduction in birth weight is seen when glucocorticoids are administered at the end of pregnancy (Nyrienda *et al,* 1998) with glucocorticoids affecting placenta size also (Gunberg, 1957).

Glucocorticoids and tissue maturation

Glucocorticoid receptors (GR) are expressed in most foetal tissues and the placenta from an early stage (Cole *et al,* 1995). Mineralocorticoid receptors are not expressed until a later stage of pregnancy (Brown *et al,* 1996).

Birth weight and foetal programming

A low birth weight has often been related to common cardiovascular and metabolic disorders seen in adult life; e.g. hypertension and type II diabetes (Barker *et al,* 1993). It is thought that exposure of the foetus to glucocorticoids may account for this (Barker *et al,* 1993).

Physiology: placental 11β-hydroxysteroid dehydrogenase type II (11β-HSD2)

Foetal glucocorticoid levels are much lower than maternal levels (Beitens *et al,* 1973). The mechanism for this is thought to be due to action of 11β-HSD2 which is expressed in the placenta. 11β-HSD2 creates a barrier to maternal glucocorticoids so only a small proportion of maternal glucocorticoids can reach the foetus (Benediktsson *et al,* 1997).

11β-HSD2 and birth weight

A deficiency in maternal 11β-HSD2 leads to overexposure of the foetus to glucocorticoids and increases the susceptibility to several diseases in later life (Edward *et al,* 1993). A strong negative correlation has been seen between placental 11β-HSD2 levels and low birth weight (Benediktsson et al, 1993).

1.13.2 Glucocorticoid programming and behaviour

Glucocorticoids are however very important in the developing CNS (Meaney *et al,* 1996). The HPA axis is very sensitive to prenatal glucocorticoids and perinatal glucocorticoid exposure permanently increases basal plasma corticosterone levels in rats (Levitt *et al,* 1996); this is thought to be due to a reduction in GR and MR receptors in the hippocampus. Prenatal glucocorticoids can also affect the developing dopaminergic system (Diaz *et al,* 1997). Stressful events in the second trimester of human pregnancy are associated with increased incidence of schizophrenia (Koenig *et al,* 2002). Hyporesponsiveness of the HPA axis to stressors in pregnancy is another mechanism by which the fetus may be protected from adverse programming actions of exposure to excess glucocorticoid.

1.14. Aims of this thesis

It is known then that the HPA axis is hyporesponsive during late pregnancy to emotional, physical and metabolic signals such as orexin-A. This reduced responsiveness is known to be due to reduced responsiveness of the pPVN CRH/AVP neurones since i.c.v. administration of orexin-A failed to activate them during late pregnancy (Brunton *et al,* 2003). Part of this reduced responsiveness is known to be due to opioid inhibition on the pPVN CRH/AVP neurones or their inputs (Brunton *et al,* 2005), or due to inhibition by neurosteroid at the later stages of pregnancy (Russell & Brunton, 2005). Oxytocin neurone responses are also suppressed by allopregnanolone and endogenous opioids.

Several questions however remain unanswered;

- There are very close relationships between feeding, metabolism and glucocorticoid secretion. Glucocorticoids are a key requirement for responding to reduced energy stores. Corticosteroids stimulate feeding and insulin secretion. Insulin-induced hypoglycaemia activates the orexin neurones in the LHA (Cai *et al,* 2002) and HPA axis responses to orexin A are known to be reduced during late pregnancy (Brunton *et al,* 2003). Insulin itself has been shown to activate the HPA axis in male rats (Plotsky *et al,* 1985). It is not known if insulin activates the HPA axis in female or in late pregnant rats.
- Ghrelin is another metabolic peptide that plays a key role in the control of food intake and is also known to activate the HPA axis. Ghrelin is thought to exert its orexigenic action via the ARC. It increases Fos expression in

NPY/AgRP neurones. Central ghrelin neurones also terminate on orexin neurones in the LHA and i.c.v. ghrelin stimulates orexin-expressing neurones (Lawrence *et al,* 2002). Since ghrelin activates the HPA axis it is interesting to know if it will do so in female rats and during late pregnancy.

- Ghrelin concentrations in blood increase before meals and are reduced by food intake. Concentrations of ghrelin are inversely related to those of insulin. Changes in the central effect of ghrelin on the HPA axis in pregnancy, without any change in insulin action, may give an indicator of whether insulin actions on the HPA axis are exerted via central ghrelin.
- It is known that the HPA axis is hyporesponsive to centrally administered orexin-A in late pregnancy. Endogenous opioids inhibit CRH/AVP responses following cytokine challenge during late pregnancy by acting pre-synaptically on noradrenergic inputs to the CRH neurones (Brunton *et al,* 2005). It is not known whether endogenous opioids would interfere with orexin signalling to the pPVN CRH/AVP neurones in late pregnancy.
- No differences between virgin and pregnant rats were found for the stimulatory effects of orexin-A on behaviours reflecting activation of centrally projecting CRH neurones or appetite. The loss of HPA axis responses to orexin in pregnancy is thus not a result of a general loss of central responses to orexin-A. Changes in patterns of hypothalamic neurone activation by orexin in late pregnancy may be expected, which may illuminate the circuitries involved in HPA axis and appetite regulation.
- Orexin signals to the HPA axis in part via the NPY neurones (Jaszberenyi *et al,* 2000) and ghrelin exerts its effects via the ARC NPY neurones. NPY is

one half of the primary population of neurones in the ARC that controls energy homeostasis. It is also known to activate the HPA axis. The effect of NPY on the HPA axis in late pregnant rats is not known or indeed if endogenous opioids interfere with NPY signalling to the pPVN CRH and AVP neurones.

- Changes in patterns of activation of hypothalamic neurones during late pregnancy could explain alterations in feeding and neuroendocrine stress response patterns.
- Changes in NPY actions on oxytocin neurones in late pregnancy are not known, though responses to other stimuli are reduced. If activity of the oxytocin system after centrally administered NPY is not increased, this may be the result of the effects of endogenous opioids on the oxytocin system.

Major adjustments in metabolic regulation during pregnancy helps ensure a successful outcome. These include increased appetite and reduced energy expenditure during exposure to stress as a result of reduced neuroendocrine stress responses.

The aim of this thesis is to investigate attenutated HPA responsiveness in pregnant rats, particularly in relation to actions of neuropeptides that regulate appetite. This includes reduced stress responses to neuropeptides that stimulate appetite which we know includes orexin but also to investigate the responses of ghrelin, NPY and insulin which normally also activate the HPA axis. Suppression of the HPA axis responses in pregnancy would help maintain a positive energy balance, as a result of reduced glucocorticoid release with stress.

Pregnancy changes also include suppressed activation of oxytocin neurones which aids prevention of premature birth.

This thesis will also investigate the effect of NPY, another orexigenic peptide and investigate if reduced responses if any on oxytocin and HPA axis activation are due to activation of inhibitory opioid mechanisms in the brain. The principal techniques used in the thesis are *in situ* hybridisation, immunohistochemistry, radioimmunoassay and behavioural analysis of eating, drinking and grooming behaviour.

GENERAL METHODS

2.1 Animals

Female Sprague Dawley rats were used (Bantin and Kingman, Hull, UK or Harlan, Oxon, UK) with an initial body weight 260-290g. They were housed in groups of 5-6 and maintained on a 12:12h light dark cycle (lights on at 07:00h) under standard conditions of temperature and humidity with food and water available *ad libitum*. The rats were given 1-2 weeks acclimatisation to animal facilities prior to any experimental procedures or mating. The rats in the pregnant groups were mated overnight with a sexually experienced male and pregnancy confirmed by the presence of a vaginal plug of semen the following morning (day 1 of pregnancy). All procedures were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986. The rats were housed individually after surgery.

2.2 Anaesthetic

For recovery experiments, rats were anaesthetised by inhalation of halothane (3% halothane in 1200ml/min of O_2), and inhalation anaesthesia was maintained throughout the surgery.

2.3 Surgery

For recovery experiments all surgery was performed under sterile conditions. All instruments were autoclaved at 121°C, fur over surgical sites was shaved and the skin sterilised with 70% alcohol and 1% w/v iodine.

2.3.1. Jugular vein cannulation

The anaesthetised rat was placed on its back on an electrically heated thermostatically controlled mat. A skin incision was made above the right clavicle and the right external jugular vein was located and the connective tissue gently teased away. When the vein was freed two ties (5.0 silk suture, Fine Science Tools, Germany) were placed loosely around the vein. The cranial tie was tightened to prevent venous return from the head. An incision was carefully made into the vein with iridectomy scissors, and a silastic cannula (bore = 0.5mm; wall = 0.25mm : Altec) 12 cm in length, filled with heparinised saline (0.9% saline, heparin 50 units/ml), was inserted. The cannula was inserted approximately 3cm into the vein to reach the right atrium until blood could be freely withdrawn into a 1ml syringe. The second tie was tightened to secure the cannula in place and prevent blood loss. A final third tie secured the cannula tightly to the vein.

The cannula was exteriorised through a small skin incision just behind the ears at the back of the neck. All wounds were closed off with silk sutures (size 2.0 Ethicon). The cannula was secured to the skin with tape and sutures. A stainless steel blocker was placed into the cannula to prevent blood loss out of the cannula.

2.3.2 Intracerebroventricular (i.c.v.) cannulation

Rats were implanted with an i.c.v. cannula 4-5 days before the experiment. In the same procedure, the anaesthetised rat was placed in a stereotaxic frame and a midline incision was made through the scalp. The connective tissue was retracted to expose lamda and bregma, the skull was levelled between bregma and lambda. The i.c.v. guide cannula (22-gauge, Bilaney Consultants Ltd, Kent, UK) was directed at the

right lateral ventricle (stereotaxic coordinates: 1.6mm lateral and 0.6mm posterior to bregma inserted to a depth of 4.6mm from the skull surface) through a burr-hole made in the skull. The cannula was secured into place with dental acrylic and two cheese head stainless steel screws (size 2mm). Finally a dummy cannula (22-gauge Bilaney Consultants Ltd, Kent, UK) was inserted into the guide cannula.

After surgery rats were handled daily, dummy caps were rinsed in 0.9% saline and loosened and tightened to familiarise the rats with the procedure prior to the day of the experiment.

2.4 *In situ* hybridisation (ISH)

ISH was developed in 1969 (Pardue *et al,*1969). It is widely used to detect the localisation of specific nucleic acid sequences within a wide range of tissue preparations. ISH uses a labelled probe to detect and localise specific DNA or RNA sequences in a tissue and relies on the ability of DNA to reanneal or hybridise with a complementary strand when exposed to the correct temperature (Gall *et al,* 1969). ISH involves a labelled (often with radioactivity) nucleic acid probe hybridising with a DNA or RNA sequence *in situ* (in cells in the tissue of interest) so that the location of the sequence of interest can be detected in the tissue (Sambrook *et al,* 1989).

ISH indicates the presence of a particular DNA or RNA sequence. It is the only procedure that allows the location of the sequence to be studied (Polak *et al,* 1990). The general procedure for ISH involves fixing samples of tissue onto a glass slide, the material is then treated with chemicals to permeabilise the tissue and denature the mRNA so that the probe can readily hybridise (Terenghi *et al,* 1990). A complementary nucleic acid probe is prepared and labelled usually with

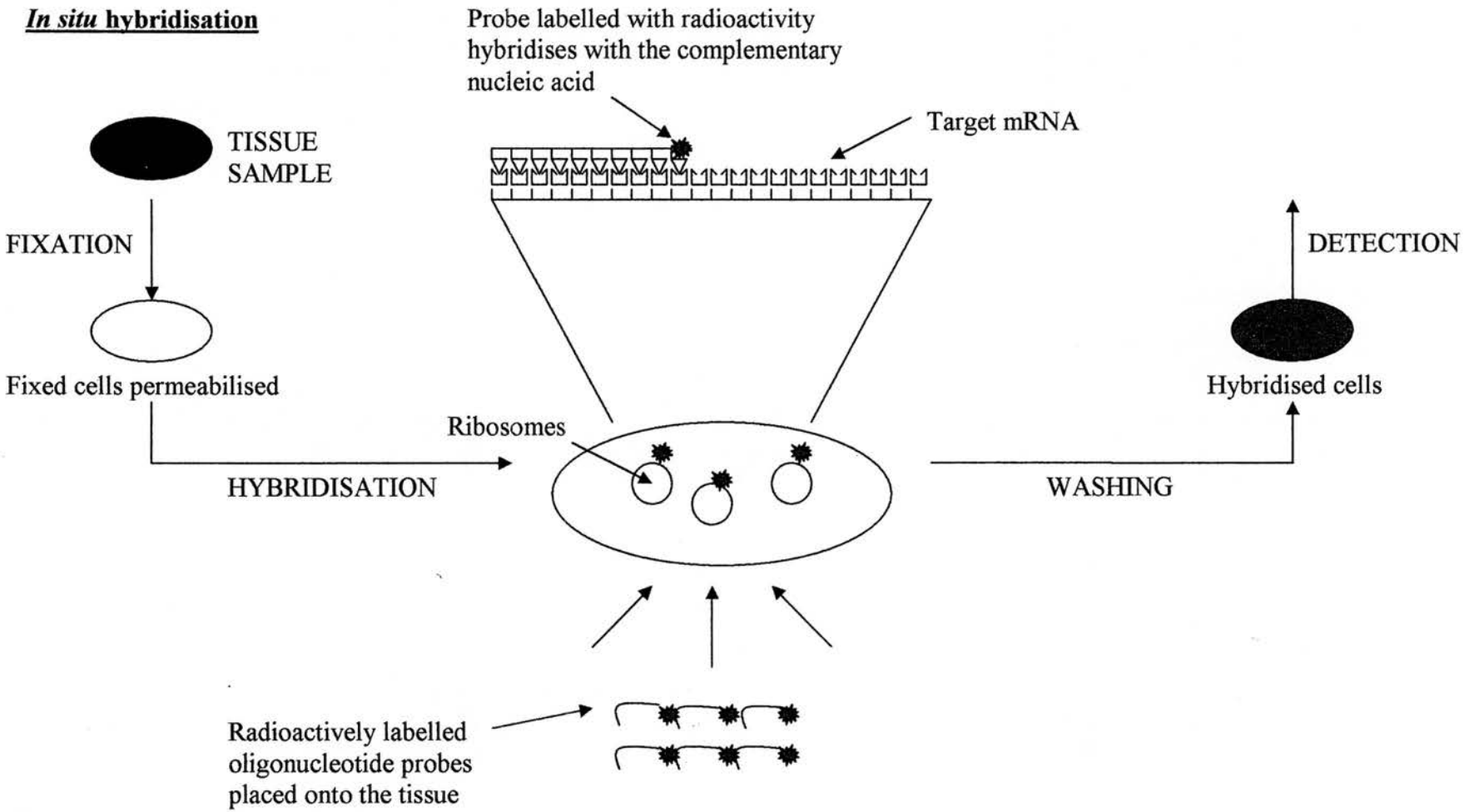


Figure 2.1: An overview of the procedures involved in *in situ* hybridisation

radioactivity. A hybridisation solution is then placed onto the slide so that the probe can hybridise with the sequence of interest. The excess probe is washed away and then autoradiography is used to detect the location of the probe. The procedure is summarised in figure 2.1. All equipment and consumables used in the process must be kept sterile to avoid contamination, especially from RNAase. Once the probe is labelled a volume containing at least 300,000 counts per minute are applied to each slide (for quantitation see section 2.4.2.vii). Various controls are used to ensure validation of the *in situ* protocol used. These are as follows. Performing the ISH using no probe, to ensure that there is no non-specific binding. A sense probe complementary to the anti-sense oligonucleotide probe is also used as a negative control to ensure that the anti-sense oligonucleotide hybridises specifically with the mRNA of interest. Radioactivity microscales also exposed to films enable ascertaining that specific labelling is in the linear portion of the standard curve of radioactivity against film or emulsion grain density. This enables calculation of the concentration of bound radio-ligand. Sections can also be pre-treated with RNAase as an additional control to check that the probe is bound to RNA. These controls were not performed in these studies as all probes used were previously characterised (Jingami *et al,* 1985; Majzoub *et al,* 1983) and quantification was relative between experimental and control groups all processed in the same ISH together.

2.4.1. Materials

(i) Microscope Slides

All sections used for ISH were mounted on slides (Polysine; BDH, Merck House, Poole, Dorset, UK). Marker sections to determine the location of areas of interest in

the brain were mounted on slides (super premium twin-frost; BDH, Merck House, Poole, Dorset, UK) dipped in a subbing solution composed of 2.25g gelatine and 0.23g chromic potassium sulphate dissolved in 1000ml ddH_2O. Slides were left to dry overnight.

(ii) Glassware and Plasticware

Glassware and metal slide racks were wrapped in foil and sterilised by baking in an oven overnight at 200°C. Plasticware (eppendorf tubes/pipette tips) was autoclaved at 121°C. Items unsuitable for autoclaving were washed thoroughly and sprayed with an RNA/DNAase inhibitor (RNAase away spray, Sigma).

(iii) General Solutions

All solutions were autoclaved and sterilised at 121°C prior to use.

DEPC-treated water

0.1 % diethyl pyrocarbonate [Sigma]

1000ml ddH_2O

0.1M Phosphate buffered saline (PBS); pH 7.2-7.4

11.5g di-sodium hydrogen orthophosphate (Na_2HPO_4) [BDH]

2.72g sodium dihydrogen orthophosphate 2-hydrate

($Na_2HPO_4.2H_2O$) [BDH]

8.5g sodium chloride (NaCl) [BDH]

1000ml DEPC treated water

20x standard Sodium Citrate (SSC)

175.4g sodium chloride (NaCl) [BDH]

88.2g tri-sodium citrate [Sigma]

1000ml ddH_2O

To make 1x SSC solution 50ml of stock solution and 950ml ddH_2O was used.

4% Paraformaldehyde in 0.1M PBS; pH 7.2-7.4

10g paraformaldehyde [Sigma]

250ml 0.1M PBS

Triethanolamine (TEA/acetic anhydride solution)

2.98 ml triethanolamine [Sigma]

0.5ml acetic anhydride [Sigma]

840µl concentrated hydrochloric acid [BDH]

200ml ddH_2O

(iv) Hybridisation Buffer components

Hybridisation buffer was prepared from 12 different components listed below. After preparation these components were added together to make hybridisation buffer (Table 2.1)

5M Sodium Chloride (NaCl)

14.6g NaCl [BDH]

50µl DEPC ddH_2O

Tris pH 7.6

2.98g Trizma base [Sigma]

20ml sterile ddH_2O

pH to 7.6

Denhardts Solution [Sigma]

Ethylenediaminetetraacetic acid (EDTA) 250mM

23.26g EDTA [Sigma]

250ml sterile ddH_2O

pH to 7.8

Dextran Sulphate

12.5g dextran sulphate [Sigma]

25ml DEPC H_2O

Sodium Pyrophosphate (NaPPI)

0.5g NaPPI [Sigma]

10ml sterile ddH_2O

Yeast tRNA

100mg yeast tRNA [Sigma]

4ml DEPC H_2O

Yeast total RNA

60mg yeast total RNA [Sigma]

3ml DEPC H_2O

Poly (A)

15mg Poly (A) [Sigma]

1ml DEPC H_2O

Formamide (50%) [Sigma]

1M Dithiothreitol (DTT)

154mg DTT [Sigma]

1ml DEPC H_2O

Salmon testes DNA

50mg salmon testes DNA [Sigma]

5ml sterile ddH2O

Hybridisation Buffer recipe

Reagents	Volume of solution added
5M NaCl (292mg/ml)	12ml
Tris 7.6 (149mg/ml)	1ml
Denhardts Solution	1ml
EDTA 250mM (232.6mg/ml)	400µl
Dextran Sulphate (500mg/ml)	5ml
NaPPI (50mg/ml)	500µl
Yeast tRNA (35mg/ml)	200µl
Yeast total RNA (20mg/ml)	250µl
Salmon testes DNA (10mg/ml)	1ml
Poly (A) (15mg/ml)	330µl
Formamide (50%)	20mls
DTT (154mg/ml)	550µl *

TABLE 2.1: Recipe for 50ml of hybridisation buffer.

All components were added to a sterile falcon tube.

* DTT was added immediately prior to use when the radioactive material was added also.

(v) Probes

There are four main different types of probes that can be used, double and single stranded DNA probes, synthetic oligonucleotide probes and single stranded RNA probes; there are advantages and disadvantages to using each of these (Agarwal *et al,* 1981). Synthetic oligonucleotide probes have good penetration properties, are commercially available and relatively inexpensive (Beesley, 2001). Synthetic oligonucleotide probes (MWG-Biotech UK Ltd.) were used throughout in the studies described in this thesis. These probes are short, single stranded pieces of DNA which are complementary to a designated segment of the mRNA of interest, and as such will seek out and bind to the mRNA, similar to an antibody binding to an antigen

(Das *et al,* 1983). These oligonucleotides can be produced by an automated DNA synthesiser in a few hours and obtained within 3-4 days (Egeland *et al,* 2005).

2.4.2. The Protocol

(i) Tissue Collection

The rats were killed by conscious decapitation. Brains (transected at the rostral pons) and brainstems were rapidly removed, placed on aluminium foil and frozen immediately on dry ice before being stored in pre-labelled plastic bags at -70°C. Coronal brain sections (15µm) containing hypothalamic PVN were cut using a cryostat at -16°C. The location of all areas of interest was confirmed using the marker slides (sections were stained with toludine blue and examined under a light microscope) with reference to a stereotaxic rat brain atlas (Konig 1963).

(ii) 3'end labelling of the oligonucleotide probe

Deoxyribonucleoside triphosphate labelling of the 3'-OH double or single stranded DNA with radioactively labelled nucleotides was catalysed by the terminal deoxynucleotidyl transferase (TdT) enzyme (Kumar *et al,* 1988). A water bath was heated to 37°C for the labelling reaction mixture below, which was put into a sterile eppendorf:

27µl sterile ddH_2O

10µl 5 x TdT tailing buffer **[Promega]**

5µl (~ 2MBq) ^{35}S dATP **[Perkin-Elmer]**

(calculated using documentation with ^{35}S)

2µl Probe (working dilution 10pmol/µl) **[MWG-Biotech]**

1µl TdT enzyme (25 units/µl) **[Promega]**

The reaction mixture was incubated in a sterile eppendorf at 37°C for 1-2 hours and cooled on ice to stop the reaction.

(iii) Purification using Spin Columns

The QIAGEN quick nucleotide removal kit (Qiagen,West Sussex) is designed for the 'clean up' of radioactive DNA fragments and oligonucleotides $\geq$17 nucleotides. It also ensures removal of primers < 10 bases, enzymes, salts and incorporated nucleotides.

500µl of buffer PN (from the kit) was added to the 50µl of reaction sample and placed into a spin column. The spin column was centrifuged for 1 min @ 6000rpm. The centrifuged spin column was then transferred into a clean 2ml collection tube and the radioactive flow-through discarded. 500µl of buffer PE (from the kit) was added to the collection tube and centrifuged for 1 min @ 6000rpm. The radioactive flow through was again discarded and the procedure repeated with another 500µl of buffer PE using the same 2ml collection tube. The flow through was discarded and then centrifuged again for an additional 1 minute at 13,000 rpm. The spin column was then placed into a clean 1.5ml eppendorf and 50µl elution buffer (EB) added to the centre of the spin column so that it covered the resin filter. It was left to stand for 1 minute and then centrifuged for a further 1 minute @ 13,000 rpm. A 1µl sample was removed from the eppendorf tube and together with 3.5ml of scintillation fluid (Ultima Gold) was counted using a β-counter.

(iv) Tissue Fixation

Slides that were selected for ISH were removed from the freezer and allowed to reach room temperature for about 1-2 hours.

The slides were placed in racks and fixed in 4% paraformaldehyde in 0.1M PBS (pH 7.2-7.4) for 10 minutes. They were then washed twice in 0.1M PBS (5 minutes each time) followed by acetylation in triethanolamine/acetic anhydride solution for 10 minutes. The slides were finally washed in sterile ddH_2O and dehydrated in an ascending alcohol series through 70%, 95% and 100% ethanol (3 minutes each), washed in chloroform for 3 minutes and then dehydrated in 100% ethanol and partially rehydrated in 95% ethanol for 3 minutes. The slides were then thoroughly air-dried.

(v) Hybridisation

Humid incubation chambers using plastic sandwich boxes lined in the base with moist (ddH_2O) filter paper were prepared. Slides were arranged on a glass platform. The labelled probe was diluted in hybridisation buffer, so that a 90µl aliquot of buffer diluted probe contained 300,000 cpm of activity. DTT was added (1µl/90µl of hybridisation buffer) to reduce background. 400µl of hybridisation buffer containing the labelled probe and DTT was pipetted onto each section on each slide and a sterile parafilm "coverslip" was positioned over the sections. The incubation chambers were then sealed and incubated at 37°C overnight.

(vi) Post-Hybridisation Washes

3 beakers containing 1 x SSC were set up at room temperature. Using forceps the Nescofilm coverslip was removed and the slides moved back and forth in the SSC slide rack. The water bath was heated to an appropriate temperature, relevant to the length of the probe – the temperature is usually 20°C below that of the melting temperature of the probe used; e.g. if the melting temperature of the probe is 75°C then the temperature of the washes is 55°C (Jingami *et al,* 1985). The slides were

rinsed in a rack for 4 x 15 minute washes at 55°C and then 2 x 30 minute washes in 1 x SSC at room temperature. The slides were finally rinsed in ddH_2O and left to dry.

(vii) Dipping slides in autoradiographic emulsion

Slides were dipped in autoradiographic emulsion to detect the location of the hybridised mRNA of interest. The emulsion (Ilford, K.5 emulsion in gel form, Knutsford, Cheshire, UK) was warmed in a waterbath at 43°C until molten. Under safelight conditions the slides were dipped in the emulsion and air dried overnight. When the slides were dried they were transferred to slide boxes (under safelight conditions) containing silica pouches. Slide boxes were sealed with electrical tape and wrapped in tin foil and stored at 4°C for the required exposure time. The optimum exposure time was determined using ^{14}C microscales to generate a standard curve. The standard curve enables calculation of the concentration of bound radioligand. A sigmoidal standard curve is generated and the optimum exposure time is chosen when radioactivity signal from the hybridised tissue is on the steepest part of the curve. Again these controls were not performed in these studies as exposure times were previously determined. Once exposed for the desired time the slides were developed under safelight conditions (Kodak D-19, Sigma, Poole, UK) and fixed (Hypam rapid fixer, Ilford, Knutsford, Cheshire, UK). The sections were then counterstained using haematoxylin and eosin and dehydrated in a graded ethanol series, before being cleared in xylene and coverslipped using DePex mount.

(viii) Quantifying Hybridised Cells

The amount of hybridised mRNA in a brain area was assessed using a light microscope (Wang 6000 series microscope x20 magnification) connected to a CCD

video camera and the NIH image analysis system (Version 1.62) on an Apple Macintosh computer.

Emulsion-dipped slides were used for cellular analysis of mRNA (objective magnification x20). The first method of analysis involved counting the number of positive expressing cells in a nucleus (of known area). A positive cell was defined as one with more overlying silver grains than the mean over an area equivalent to $1cm^2$ 10 cells lateral to the area of interest (background + 3 s.d.).

A second method of analysing emulsion dipped slides was to measure silver grain density over cells in the region of interest using the NIH program. A fixed circular counting frame was set for identification of a labelled cell and for background readings. Silver grain density measurements were made over 5-6 cells in the nucleus per side of PVN and over an equivalent area in the nearby background.

When measuring AVP mRNA expression often the staining was so dense that the area of the PVN was measured and the amount of silver grain within that area measured. Background measures (of known area) adjacent to the PVN were also measured and the area of the PVN normalised to 1.

2.5 Immunohistochemistry (IHC)

IHC was first used in 1941 (Coons *et al,* 1950). It is a technique for identifying cellular or tissue constituents by means of an antigen-antibody interaction. An antibody is used to link an antigen specifically to a stain that can be readily seen with a microscope (Coons *et al,* 1950). Many macromolecules can be successful antigens for raising antibodies, and these antibodies can either be polyclonal or monoclonal. Monoclonal antibodies are much more specific (Hsu *et al,* 1981). Fixatives are

needed to preserve protein in cells; either the tissue is fixed *ex vivo* or the deeply anaesthetised animal is perfused with a fixative via the circulatory system. The antigen can be localised either by direct labelling using 1 step, indirect labelling using 2 steps or indirect labelling using two-steps and signal amplification. In the present studies the two-step indirect method was used. In this procedure an unlabelled primary antibody binds to the antigen and is visualised by a labelled secondary antibody targeted against antibody from the animal in which the first antibody was raised, in this case rabbit (Park *et al,* 1982).

Fos immunohistochemistry

Fos is the protein product of the immediate early gene *c-fos* and can be detected by immunohistochemistry using either an indirect method as described by Shu (Shu *et al,* 1988), or the avidin-biotin complex (ABC) (Srisawat *et al,* 2000). In the present study Fos protein was localised using a rabbit affinity purified polyclonal antibody: *c-fos* (Ab-2; Oncogene Research Products). Fos immunoreactivity was visualised using a labelled secondary antibody: goat anti rabbit IgG peroxidase (Vector Laboratories). Diaminobenzidine (DAB) was used as the substrate for the enzyme reaction, which converts a colourless chromagen into coloured end products. The reaction was enhanced by nickel-glucose oxidase (Elias *et al,* 1987). The procedure is summarised in Figure.2.2.

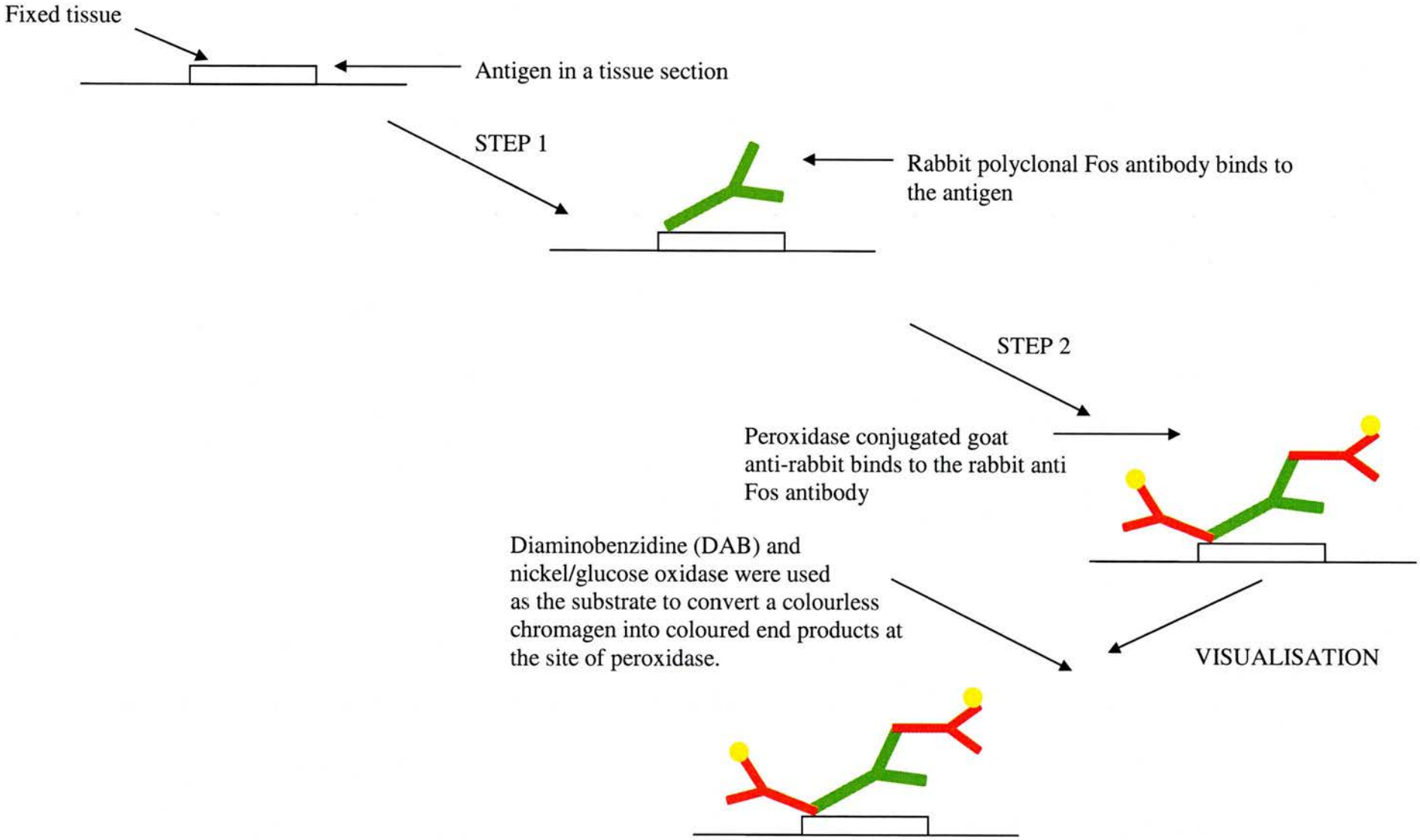


Figure 2.2 The steps in Fos immunohistochemistry

2.5.1 Materials

(i) Microscope slides

Super premium twin-frost slides (BDH, Merck House, Poole, Dorset, UK) were dipped in a subbing solution composed of 5g gelatine, 0.46g chromic potassium sulphate mixed with 1000ml ddH_2O. Slides were left to dry overnight.

(ii) General Solutions

1M Phosphate Buffer (PB) pH 7.2-7.4

115g disodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$) [BDH]

27.2g sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) [BDH]

1000ml dH_2O

0.1M Phosphate Buffer-Triton-X (PB-T)

0.2% (v/v) Triton-X [Sigma]

0.1M PB

4% paraformaldehyde

40g paraformaldehyde [Sigma]

500ml 0.1 M PB

500ml dH_2O

Heparinised Saline

9g NaCl [BDH]

129mg Heparin [Sigma]

1000ml dH_2O

Hydrogen peroxide/methanol solution (to inactivate the endogenous peroxidase)

20% (v/v) methanol [BDH]

1.5% (v/v) hydrogen peroxide [Sigma]

80% (v/v) 0.1M PB

Preincubation Buffer

1% (v/v) normal sheep serum [Sigma]

PB-T

Primary Antibody Solution

0.2% (v/v) triton-X [Sigma]

1% (v/v) normal sheep serum [Sigma]

Primary Antibody (1:1000) [Oncogene Biosciences]

0.1M PB

Second Antibody Solution

Biotinylated anti-rabbit IgG (10µl/ml) [Vector]

Normal Goat Serum (30µl/ml) [Vector]

PB-T

Antibody-Antigen Complex

Avidin DH (250µl/ml) [Vector]

Biotinylated horseradish peroxidase (250µl/ml) [Vector]

PB-T

0.2M Sodium Acetate Buffer pH 6.0

27.2g Sodium Acetate (CH_3COONa) [Sigma]

1000ml dH_2O

0.1M Sodium Acetate Buffer

500ml sodium acetate buffer

500ml dH_2O

Nickel-DAB solution

5g nickel ammonium sulphate	[Sigma]
400mg glucose	[BDH]
80mg ammonium chloride	[Sigma]
50mg diaminobenzidine (DAB)	[Sigma]
3mg glucose oxidase*	[Sigma]

100mls 0.2M sodium acetate buffer

100mls dH_2O *glucose oxidase was added 30 seconds prior to use

2.5.2 Protocol for floating sections

(i) Tissue collection

At the end of the experiment the rats were killed by transcardial perfusion. The rats were deeply anaesthetised with pentobarbitone (60mg/ml, 0.7ml/kg), delivered via an intra-peritoneal injection. The chest was opened to expose the heart and the rats were perfused with ~ 200 ml heparinised saline (50ml, 20U/ml 0.9% saline) via the left ventricle and ascending aorta, followed by ~ 300 ml of 4% (w/v) paraformaldehyde. The brain, brainstem and pituitary were removed and stored progressively in post-fix solution (1:1 mix of 4% paraformaldehyde in 0.1 M PB and 15% sucrose in 0.1M phosphate buffer) and then transferred to 30% sucrose solution in 0.1M phosphate buffer before being frozen and stored at -70°C.

Coronal microtome brain sections (52μm) through the hypothalamus were collected in sterile pots and stored in cryoprotectant (200ml glycerol, Sigma; 300ml ethylene glycol, Sigma; 500 ml 0.2M PBS) at -20°C until required for use.

(ii) Primary Antibody Preparation

Sections stored in cryoprotectant were removed from the freezer and allowed to reach room temperature. They were transferred to sterile petri-dishes in a small sieve and given 3 x 5 min washes and 2 x 10 min washes in 0.1M PB with 0.2% (v/v) PB-T. Sections were then washed in 0.1M PB for 5 minutes followed by a 15 minute wash in the hydrogen peroxide/methanol solution to deactivate the endogenous peroxidase in the hypothalamus. In order to wash out the methanol and aid impregnation of the brain tissue the sections were washed in 0.2M PB with 0.2% (v/v) PB-T. They were then washed in the pre-incubation solution for 1 hour before being placed in small vials containing primary antibody solution for 20-48 hrs at 4°C.

(iii) Secondary Antibody and Visualisation

After incubation the sections were washed in PB-T for 3 x 5 min and 2 x 10 min. The sections were then incubated in the secondary antibody solution for 1 hour and were then further washed in PB-T for 3 x 5 min and 2 x 10 min. The sections were further incubated for 1 hour in the peroxidase/antiperoxidase (PAP) complex. Sections were washed in PB-T for 2 x 10 min and then prepared for visualisation by washing in 0.1M sodium acetate buffer. Nickel-DAB solution was added to the sections and the reaction closely monitored. Sample sections were periodically placed on slides and examined under the microscope to track the progress of the reaction. All sections in the experiment were incubated for the same time. When staining was judged sufficient the reaction was stopped by immersing in 0.1M sodium acetate buffer for 5 minutes and sections were stored in 0.1M PB until mounted onto gelatinised slides. The slides were air dried and then further dehydrated with 50%, 70%, 80%, 90% and 95% alcohol for 5 minutes each and then washed twice in 100% alcohol and xylene

for 10 minutes each. Coverslips were coated with DePeX and placed over each slide, ensuring that all air bubbles were pressed out. The slides were left to dry overnight. To validate the protocols various measures are used. Immunohistochemistry is performed without primary antibody to ensure signal is specific; the Fos antibody used in the present study has already been well-characterised. Rats given i.p. hypertonic saline (2M, 0.5ml and killed 90 min later) were used as positive controls in every Fos experiment since this treatment reliably induces Fos expression in specific brain regions (Ludwig *et al,* 1997).

(iv) Quantitative Analysis

Immunohistochemically stained sections were examined under a microscope at x40 objective magnification, and the number of Fos positive nuclei counted on both sides in the arcuate nucleus, paraventricular nucleus, supraoptic nucleus, lateral hypothalamic area, ventromedial hypothalamus and dorsal medial hypothalamus. The slides contained three sections and were coded to ensure that cell counts were 'unbiased' and 'blind'. Mean values were calculated from the three sections on each slide and for all slides on each experimental group. Photomicrographs were taken of sections using a digital camera at x20 and x40 magnification.

2.5.3. Protocol for unfixed cryostat sections

(i) Tissue Collection

This method was used where brains were also collected and sectioned for ISH. The rats were killed by conscious decapitation and brains/brainstems were rapidly removed and frozen on dry ice. The brains/brainstems were cut into 15μm coronal sections through the region of interest using a cryostat. The sections were thaw

mounted onto gelatine coated slides and stored at -70° C until immunohistochemical processing.

(ii) Primary Antibody Preparation

Sections were removed from the freezer and allowed to reach room temperature. They were outlined with a PAP pen (Sigma Aldrich), transferred to glass staining dishes and fixed in 4% paraformaldehyde for 30 minutes at room temperature, followed by 3 x 5 minute washes in 0.1M PB. Endogenous peroxidase was deactivated with the hydrogen peroxide solution for 15 minutes. In order to wash out the methanol and aid impregnation of the brain tissue the slides were washed in 0.2M PB with 0.2% (v/v) PB-T, they were then washed in the pre-incubation solution for 1 hour. The slides were transferred to flat trays containing filter paper soaked in 0.1M PB and coated (750µl/slide) in primary antibody solution and incubated for 20-48 hrs at 4°C. Additional primary antibody solution was applied if necessary if the sections had begun to dry out.

(iii) Secondary Antibody and Visualisation

The following day primary antibody solution was drained from the slides, which were washed in PB-T for 3 x 5 min and 2 x 10 min. The slides were returned to the flat trays and coated in secondary antibody solution (750µl/slide) for 1 hour, these were drained then further washed in PB-T for 3 x 5 min and 2 x 10 min. The slides were further incubated for 1 hour in the peroxidase complex. Slides were washed in PB-T for 2 x 10 min washes and then prepared for visualisation by washing in 0.1M sodium acetate buffer. The visualisation solution was added to the sections and the reaction closely monitored. A sample slide was periodically examined under the microscope to track the progress of the reaction. When staining was judged sufficient

the reaction was neutralised by immersion in 0.1M sodium acetate buffer for 5 min. The slides were air-dried and then further dehydrated with 50%, 70%, 80%, 90% and 95% alcohols for 5 minutes each and then washed twice in 100% alcohol and xylene for 10 minutes each. Coverslips were coated with DePeX and placed over each slide, ensuring that all air bubbles were pressed out. The slides were left to dry overnight.

(iv) Quantitative Analysis

Immunohistochemically stained sections were examined under a microscope at x40 objective magnification and the number of Fos positive nuclei counted on both sides in the arcuate nucleus, paraventricular nucleus, supraoptic nucleus, lateral hypothalamic area, ventromedial hypothalamus and dorsal medial hypothalamus. A Fos positive cell was defined as a cell with a nucleus that was stained clearly darker (i.e. was black) than a background cell (see photomicrographs for comparison). The slides each contained three sections and were labelled with a coded number to ensure that cell counts were made 'blind' to treatment. Mean values were calculated from the three sections on each slide, and for all slides for each experimental group. Photomicrographs were taken of sections using a digital camera at x20 and x40 objective magnification.

2.6 Blood Samples

Jugular venous blood was collected into sterile 1ml syringes and placed into 1.5ml eppendorf tubes containing 50µl 5% EDTA. All blood samples were stored on ice until centrifugation. Blood was centrifuged at 13,000 rpm for 2 minutes. The plasma was separated and stored at -20°C until radioimmunoassay. In each experiment,

blood removed from the animal was immediately replaced with sterile 0.9% saline via the jugular cannula.

Blood glucose was measured using a Roche Accuchek Active Meter; <100µl blood was taken and applied to the test stick. Results (mmol/l) were obtained immediately.

2.7 Adrenocorticotropic hormone (ACTH) immunoradiometric assay

ACTH is a polypeptide consisting of 39 amino acids. In this case a commercially available two-site immunoradiometric kit (IDS Ltd. Sweden) was used to quantify the concentration of ACTH in a plasma sample (Hodgkinson *et al,* 1984). A two-site immunoradiometric assay involves two sets of antibodies one of which is radioactively labelled. The two antibodies combine with different immunoreactive sites on an antigen molecule and therefore detect the intact antigen.

Two antibodies were used here to combine with different sites on the ACTH molecule. One was a radioactively labelled ^{125}I sheep antibody which recognises the amino terminal of the ACTH molecule. The other was a C-terminal anti-ACTH rabbit antibody which coupled to the sheep anti-rabbit coated tubes provided in the kit. This antibody also reacts with the C-terminal region of the ACTH molecule. Following overnight incubation a 'sandwich type' complex forms between the sheep anti-rabbit antibody on the wall of the coated tubes and the second antibody. The tubes were washed twice and decanted to remove any excess ^{125}I sheep anti- ACTH. The amount of radioactivity in each tube was proportional to the concentration of ACTH in each tube. Each tube was counted in a gamma counter (Wallac) for 1 minute. The principle of the procedure is summarised in Figure 2.3. Sensitivity of the ACTH assay was calculated. Reference standards were provided as quality

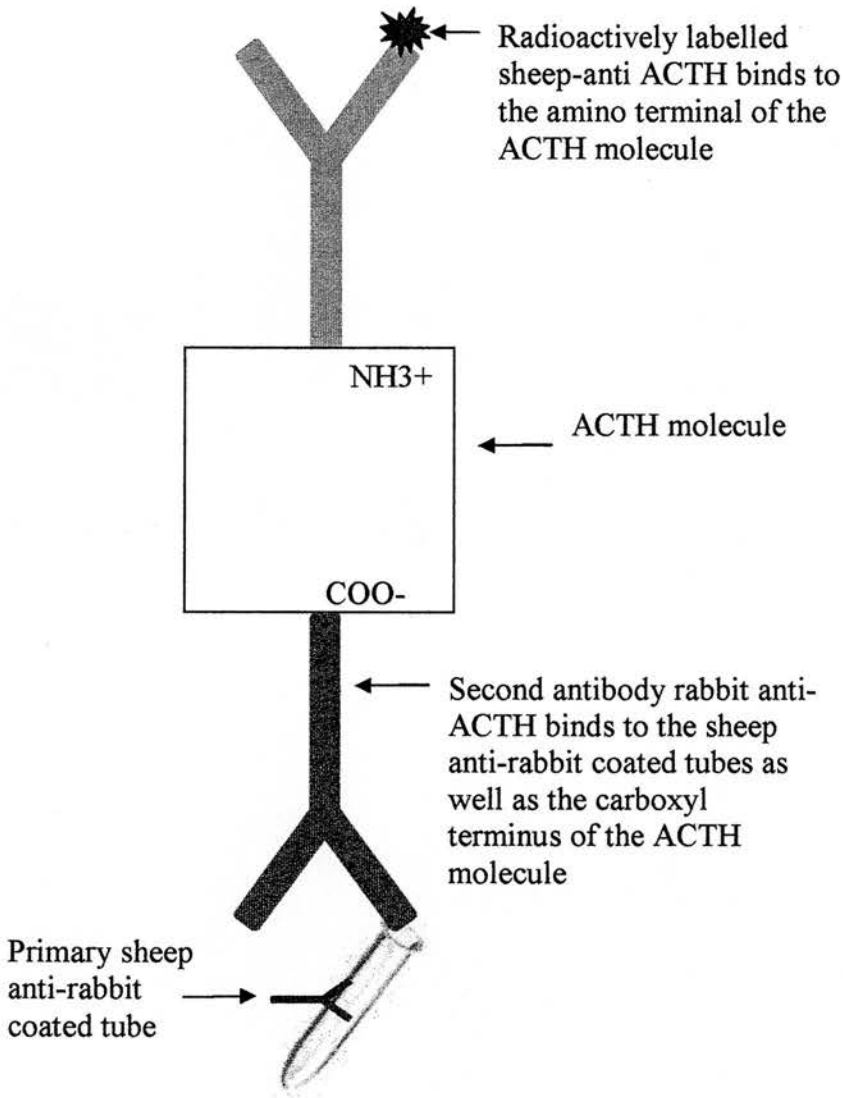


Figure 2.3 The 'sandwich' type complex in the ACTH immunoradiometric kit.

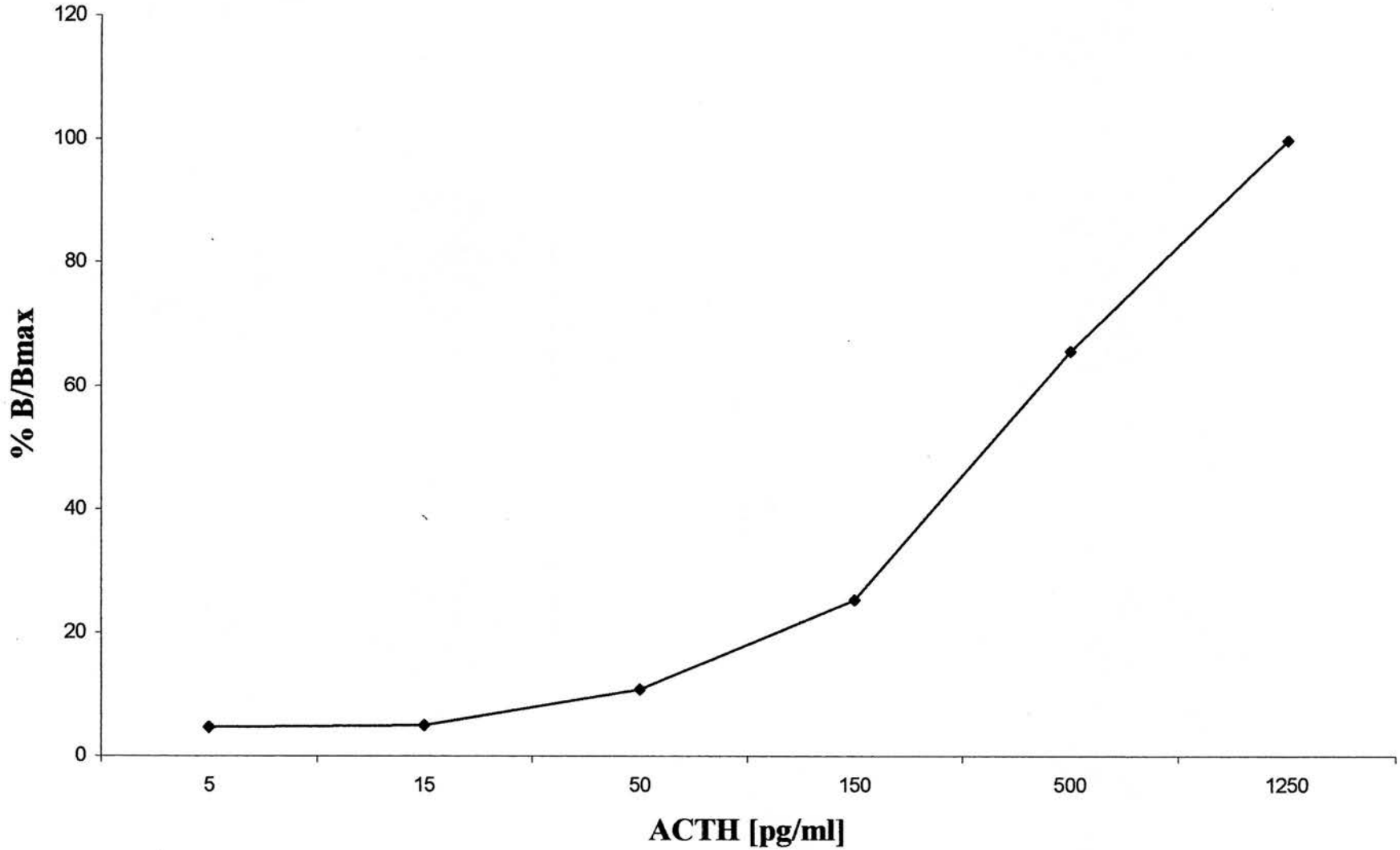


Figure 2.4: An ACTH standard curve was generated by plotting the corrected cpm (%B/Bmax) from the calibrators provided in the kit. The ACTH concentrations of the unknown samples can then be generated from the B/Bmax measurements.

controls. A standard curve was created from the concentration obtained from the calibrators, which allowed the concentration of ACTH in the plasma samples to be calculated. Reference controls I and II were provided as quality controls. The concentration of ACTH (pg/ml) in these controls was provided and this was checked with the readings obtained in the laboratory.

Wallac (Perkin Elmer, USA) software automatically generated a standard curve by plotting the average radioactivity (cpm) for each tube against the known concentration of the ACTH standards and calculated the unknown values in the plasma sample (Figure 2.4).

2.7.1 Materials

An ACTH IRMA kit (IDS Ltd., Sweden) containing:

(i) Seven standard ACTH solutions ranging from 0pg/ml – 1250pg/ml

(ii) Two reference controls containing different concentrations of ACTH

(iii) [^{125}I]-ACTH antibody (rabbit anti-ACTH)

(iv) Coated tubes: C terminal anti-ACTH coupled to sheep anti-rabbit coated tubes

(v) Wash Buffer

2.7.2 Protocol

Tubes coated internally with C-terminal anti-ACTH, coupled to sheep anti-rabbit antibody coated tubes were labelled for standard and plasma samples. Glass tubes were labelled 1 and 2 for the total counts. The calibrators, controls and ^{125}I antibody were reconstituted with distilled water. Plasma samples were defrosted and allowed to reach room temperature. These and the standards (calibrators, A-G) were

vortexed gently before use. 200µl of each standard, control and plasma sample was carefully pipetted into tubes. 200µl of the ^{125}I labelled antibody solution was added to each tube. This radioiodinated antibody binds to the amino terminal of the ACTH molecule. The second immobilised antibody reacts non-competitively with the C-terminal region of the ACTH molecule. All tubes were vortexed and incubated overnight at room temperature, to allow a 'sandwich type' complex to be formed between the sheep anti-rabbit antibody on the wall of the coated tubes and the second antibody and between the ACTH and the two anti-ACTH antibodies. The coated tubes were washed twice to remove any excess tracer, and then the liquid aspirated completely. The amount of radioactivity in each tube was proportional to the concentration of ACTH in each tube. Each tube was counted in a gamma counter (Wallac) for one minute.

2.8 Radioimmunoassay (RIA)

RIA has been used for many years in research and clinical practice in endocrinology. It was first used in 1960 (Yalow *et al,* 1960). It involves the separation of the hormone using the specificity of antibody-antigen binding, and quantitation using radioactivity (Yalow *et al,* 1960). A mixture of radioactive antigen, often ^{125}I, with antibodies directed against the antigen is used. Known amounts of unlabelled antigen are added to samples of the mixture to produce standard curves, or aliquots of the unknown sample are added. The added labelled and unlabelled antigen competes for the binding sites of the antibodies. At increasing concentrations of unlabelled antigen an increasing amount of radioactive antigen is displaced from the antibody molecule. The antibody-bound antigen is separated from the free antigen in the

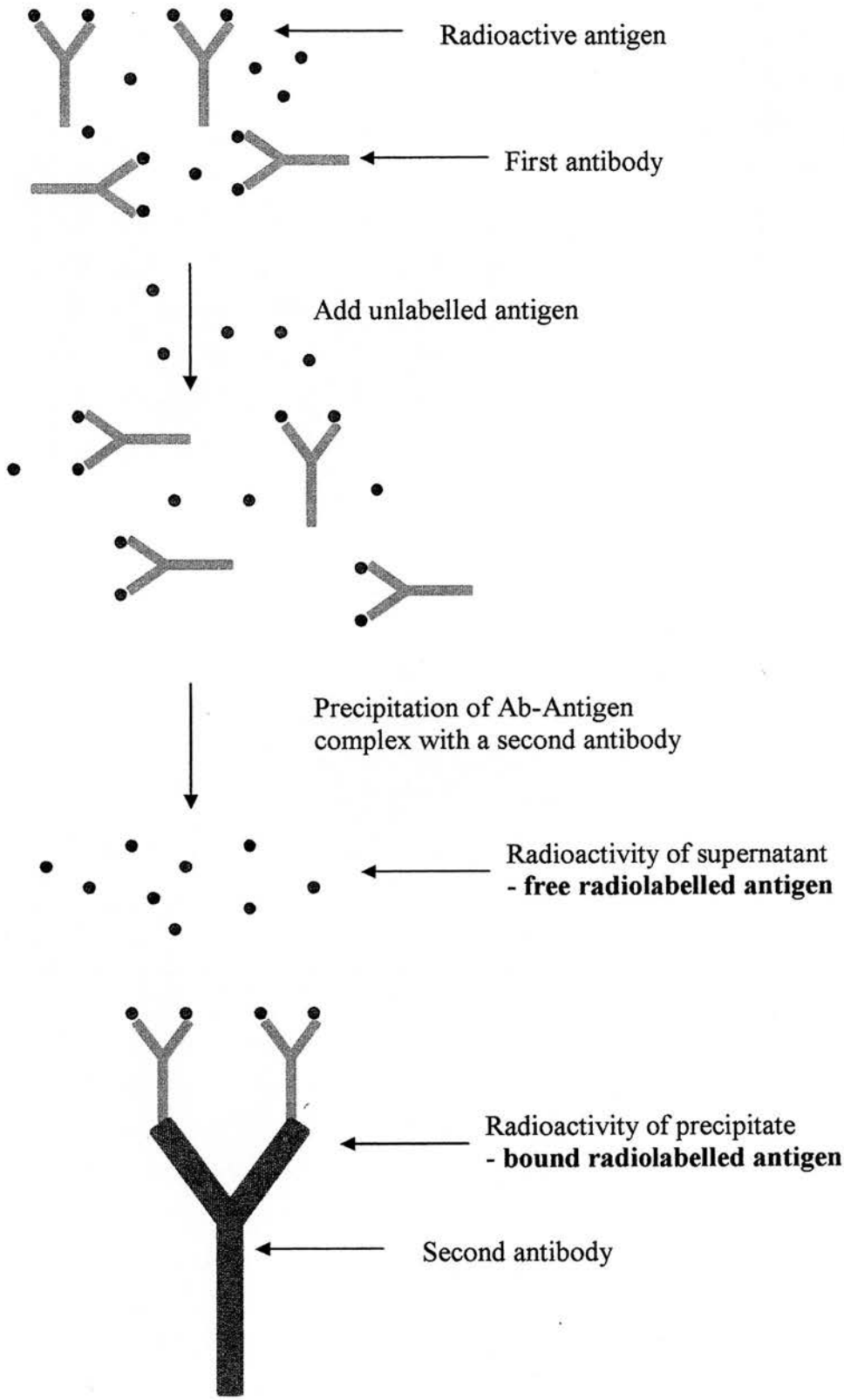


Figure 2.5: Diagram of the principle of radioimmunoassay

supernatant fluid and the radioactivity of each is measured (Pots *et al,* 1967). Concentrations of unknown antigen are then read from the standard curve.

There are several ways to separate the bound from the free antigen. One way is to precipitate the antigen-antibody complex by adding a second antibody directed against the first, for example if a rabbit antibody was used to bind to the antigen then the complex can be precipitated by adding an anti-rabbit antibody. This procedure is summarised in Figure.2.5.

2.8.1 Corticosterone Radioimmunoassay

Corticosterone is a glucocorticoid produced by the adrenal cortex in response to adrenocorticotropic hormone (ACTH) (Jones *et al,* 1987). The production of glucocorticoids is increased by stress, therefore change in plasma corticosterone concentration is an indicator of adrenal cortex secretion stimulated by ACTH. Plasma corticosterone concentration shows a circadian variation controlled by the suprachiasmatic nucleus (SCN), acting through ACTH (Takahashi et al, 2001). Plasma corticosterone concentrations were quantified using a commercially available radioimmunoassay kit (IDS Ltd.) which is a competitive assay. It uses ^{125}I-corticosterone and an anti-corticosterone antiserum to determine the concentrations of hormone in a plasma sample by the double antibody technique. It permits corticosterone measurements within the range of 0.5-62.5 ng/ml.

2.8.2. Materials

A gamma-B I^{125}-Corticosterone RIA kit containing:

(i) Assay Buffer: phosphate buffered saline

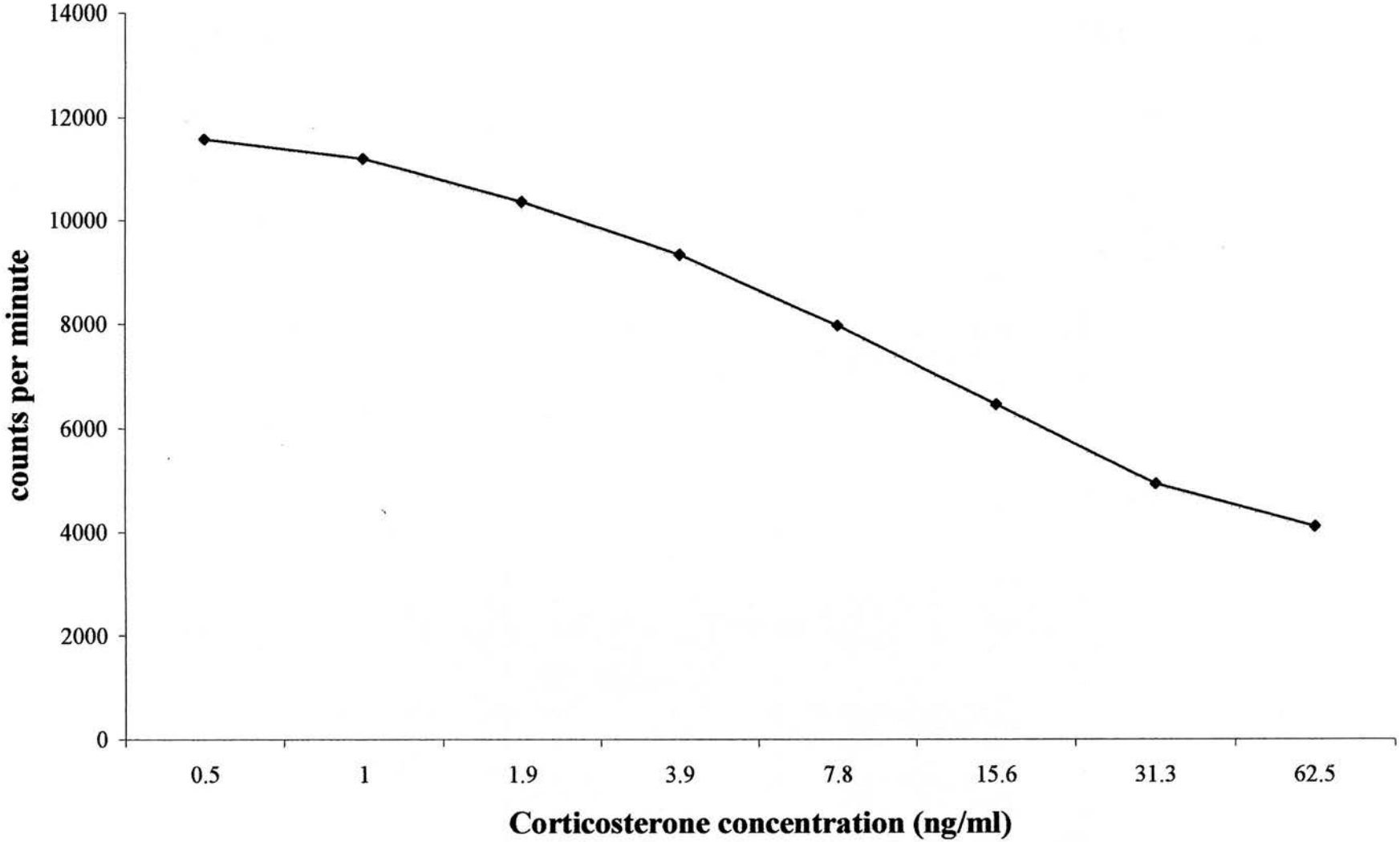


Fig 2.6 A corticosterone standard curve was generated by plotting the cpm from the calibrators provided in the kit.

(ii) Corticosterone antiserum: rabbit anti-corticosterone

(iii) [^{125}I]-corticosterone

(iv) Second antibody: goat anti-rabbit antiserum

(v) Corticosterone standard: 1000ng/ml corticosterone

2.8.3 Protocol

Eleven glass tubes were labelled A-K for a series of standards of known concentrations of corticosterone. Standards were serially diluted, ranging in concentration from 0.5 – 62.5 ng/ml. The samples and standards were added to their respective tubes (samples were diluted 1:8 in assay buffer). 100µl ^{125}I–corticosterone was added to all the tubes, which were vortexed, and 100µl of corticosterone antiserum was added to all tubes except for the total count tubes and non-specific binding tubes. The tubes were vortexed and incubated overnight at 4°C. Following incubation, 100µl of the second antibody was added to all tubes except for the total count tubes. The tubes were vortexed and incubated for one hour at room temperature. 1.0ml of saline was added to all tubes except the total count tubes. Tubes were centrifuged at 1500-1600g for 20 minutes at 2-8°C. The supernatant of each tube was then immediately carefully decanted and the rim of each tube blotted on absorbent paper. Decanting was done smoothly and carefully to avoid dislodging the pellet. The radioactivity in the tubes was counted for at least one minute in a gamma counter (Wallac). A quality control report was provided with an acceptable range of values (ng/ml). The software of the gamma counter calculated a standard curve (Fig.2.6).

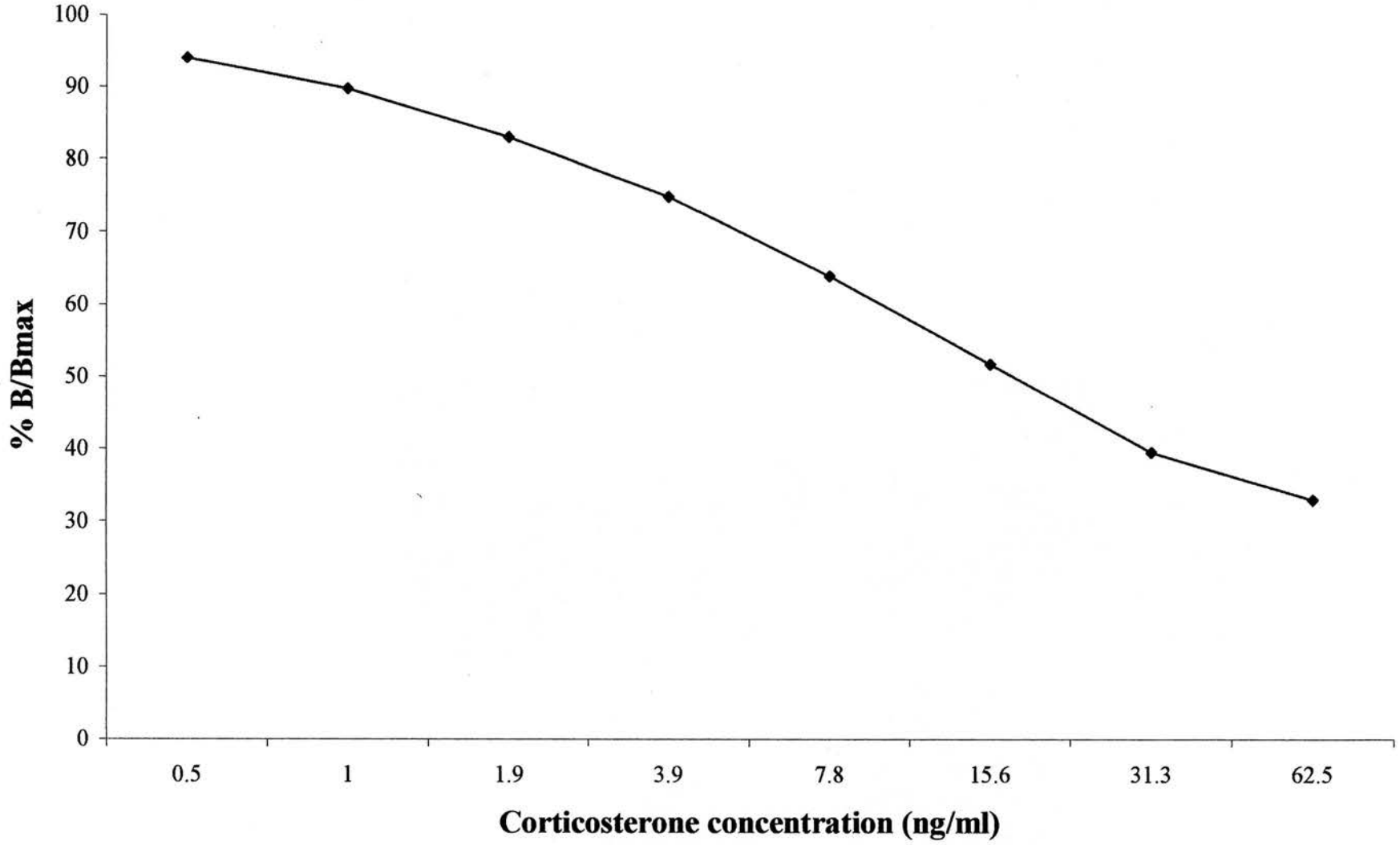


Figure 2.7 A corticosterone standard curve was generated by plotting the corrected cpm (%B/Bmax) from the calibrators provided in the kit. The concentration of the unknown samples can then be generated.

In order to calculate the sensitivity of an assay it is necessary to calculate the amount of radioactivity bound by each standard (B) as a % of the maximum binding in the assay (Bmax) which can be defined as B/Bmax. This can be quantified using the following equation.

$$\%\ B/Bmax = \left(\frac{(\text{mean cpm of standard} - \text{mean cpm of NSB*})}{(\text{mean cpm of Bmax} - \text{mean cpm of NSB})} \right) \times 100$$

* NSB = non-specific binding (binding that is not specifically bound to the antibody)

From these data a standard binding curve can be obtained (Figure 2.7). The graph can then be used to convert the sensitivity of the assay from the percentage B/Bmax to a hormone concentration.

$$\text{Sensitivity} = \frac{[\text{Bmax} - (2 \times \text{s.d. Bmax})] - \text{NSB}}{\text{Bmax} - \text{NSB}}$$

2.9. Statistics

A two-way analysis of variance (ANOVA) was used with a repeated measures ANOVA used for blood sampling experiments. Each time the ANOVA was followed by a Student Newman Keuls multiple comparison test, to locate significant differences. A p value of <0.05 was considered statistically significant. The statistical package used was Sigma Stat.

2.10. List of chemicals and Suppliers

Chemical/Material	Supplier
^{35}S dATP	Perkin-Elmer, USA
i.c.v. guide cannula	Bilaney Consultants, Dusseldorf
i.c.v. dummy cap	Bilaney Consultants, Dusseldorf
acetic anhydride	Sigma, UK
ACTH immunoradiometric kit	IDS Ltd, Berkshire
Ammonium chloride	Sigma, UK
Avidin DH	Vector
Biotinylated anti-rabbit IgG	Vector
Biotinylated horseradish peroxidase	Vector, UK
Cannula (i.v. 0.55 mm bore, 0.25mm wall)	Altec, Cornwall
Corticosterone radioimmunoassay kit	IDS Ltd, Berkshire
Denhardts solution	Sigma, UK
DePeX	BDH, Poole
Developer D-19 professional developer	Kodak, UK
dextran sulphate	Sigma, UK
Diaminobenzidine (DAB)	Sigma, UK
diethyl pyrocarbonate (DEPC)	Sigma, UK
di-sodium hydrogen orthophosphate (NaH_2PO_4)	BDH, Poole
dithiothreitol (DTT)	Sigma, UK
Emulsion gel k.5 emulsion in gel form	Ilford, Cheshire
Ethanol	BDH, Poole

ethylenediaminetetraacetic acid (EDTA)	Sigma, UK
Fixer Rapid Fixer lot # 19D0055	Ilford, Cheshire
Suture thread (2.0 gauge)	Ethicon, UK
formamide	Sigma, UK
Ghrelin	Phoenix Pharmaceuticals, USA
Glucose	BDH, Poole
Glucose oxidase	Sigma, UK
Heparin	Sigma, UK
hydrochloric acid	BDH, Poole
Hydrogen Peroxide	Sigma, UK
Insulin (human fast acting)	Novo Nordisk, UK
Iodine	Genus Express, York
Methanol	BDH, Poole
Naloxone	Tocris, UK
NPY	Tocris, UK
Nickel ammonium sulphate	BDH, Poole
Normal goat serum	Vector
Normal sheep serum	Sigma, UK
Orexin	Sigma, UK
Qiagen quick nucleotide removal kit	Qiagen, Sussex
PAP pen	Sigma, UK
paraformaldehyde	Sigma, UK
Polysine Slides	BDH Ltd, Poole
poly (A)	Sigma, UK

Primary antibody (c-fos polyclonal antibody)	Oncogene Biosciences, UK
RNAse/DNAase away spray	Sigma, UK
salmon testes DNA	Sigma, UK
Silk Suture (5.0 gauge)	Fine Science Tools, Germany
Sodium acetate	Sigma, UK
sodium dihydrogen orthophosphate ($Na_2HPO_4.2H_2O$)	BDH, Poole
sodium chloride (NaCl)	BDH, Poole
sodium pyrophosphate (NaPPI)	Sigma, UK
super premium twin-frost slides	BDH Ltd, Poole
synthetic oligonucleotide probes	MWG-Biotech Ltd, UK
TdT (terminal deoxynucleotidyl transferase)	Promega, Southampton
Triton-X	BDH, Poole
tri-sodium citrate	Sigma, UK
triethanolamine (TEA)	Sigma, UK
trizma base	Sigma, UK
Xylene	BDH, Poole
yeast tRNA	Sigma, UK
yeast total RNA	Sigma, UK

INSULIN INDUCED HYPOGLYCAEMIA

3.1 Insulin induced Hypoglycaemia

3.1.1. Insulin and the central nervous system

Insulin is a 51 amino acid polypeptide composed of two separate chains (A and B) linked by two disulphide bonds (Brange *et al*, 1993). Insulin is secreted from the endocrine pancreas in response to increasing blood glucose concentration, and circulates at levels proportional to body fat and inhibits food intake by actions on the hypothalamic NPY/AgRP neurones (Porte *et al*, 2002). It can cross the blood-brain barrier into the arcuate nucleus (ARC) (Woods *et al*, 1977), which is a critical site for sensing insulin. Information provided by these signals is passed to downstream areas including the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA), which convey information to areas such as the nucleus of the solitary tract (NTS) (Grill *et al*, 2002) to regulate meal size.

Insulin inhibits the neuropeptide Y (NPY) and Agouti related peptide (AgRP) neurones within the ARC, two peptides that potently stimulate food intake, reduce energy expenditure and promote weight gain (Suda *et al*, 1993). The orexigenic actions of NPY are mediated via activation of Y1 and Y5 receptors (Raposinho *et al*, 2004) while those of AgRP come from antagonism of melanocortin-4 receptors (Ellacott *et al*, 2004). The PVN and the LHA express receptors for both NPY and AgRP. As well as NPY and AgRP the ARC also contains neurones that produce α-melanocyte stimulating hormones (α-MSH), a peptide that reduces food intake and is derived from the precursor proopiomelanocortin (POMC). Many POMC neurones in the ARC also co-express the cocaine-amphetamine-regulated transcript (CART), which has complementary actions to α-MSH, inhibiting appetite (Volkoff *et al,* 2005). Insulin stimulates the POMC/CART neurones which innervate the same

hypothalamic areas as NPY and AgRP (Fekete *et al*, 2004). The way in which insulin works in the central nervous system (CNS) is illustrated in Fig.3.1.

Insulin receptors have been identified in the brain and shown to be concentrated in the hypothalamus (Havarankova *et al*, 1978). They have binding properties similar to those of the peripheral insulin receptor. Studies of the pattern of uptake of insulin from the blood into cerebrospinal fluid (CSF) have shown a receptor-mediated transport system as a means for regulated insulin delivery into the CNS (Baura *et al*, 1993).

3.1.2. Insulin receptors in the brain

Insulin can affect the function of the CNS by producing hypoglycaemia, although as discussed above insulin also acts directly on cells of the CNS to modify their function (Woods *et al*, 1975). In a study of the phylogeny of the insulin receptor it was shown that radioactively labelled insulin binds specifically to membrane preparations of the rat brain. There are substantial concentrations of insulin receptors in specific regions of the CNS, particularly in the rat hypothalamus (Gavin *et al*, 1972).

The insulin receptor consists of an extracellular alpha subunit involved in ligand binding and an intracellular beta subunit that transduces the insulin signal as a tyrosine kinase (Fig 3.2). Binding of insulin to the alpha subunits causes the beta subunits to auto-phosphorylate so activating the receptor. The activated receptor then phosphorylates a number of intracellular proteins. Several intracellular proteins have been identified as phosphorylation substrates for the insulin receptor. Two classical ones are the insulin receptor substrate 1 (IRS-1) and the insulin receptor substrate 2

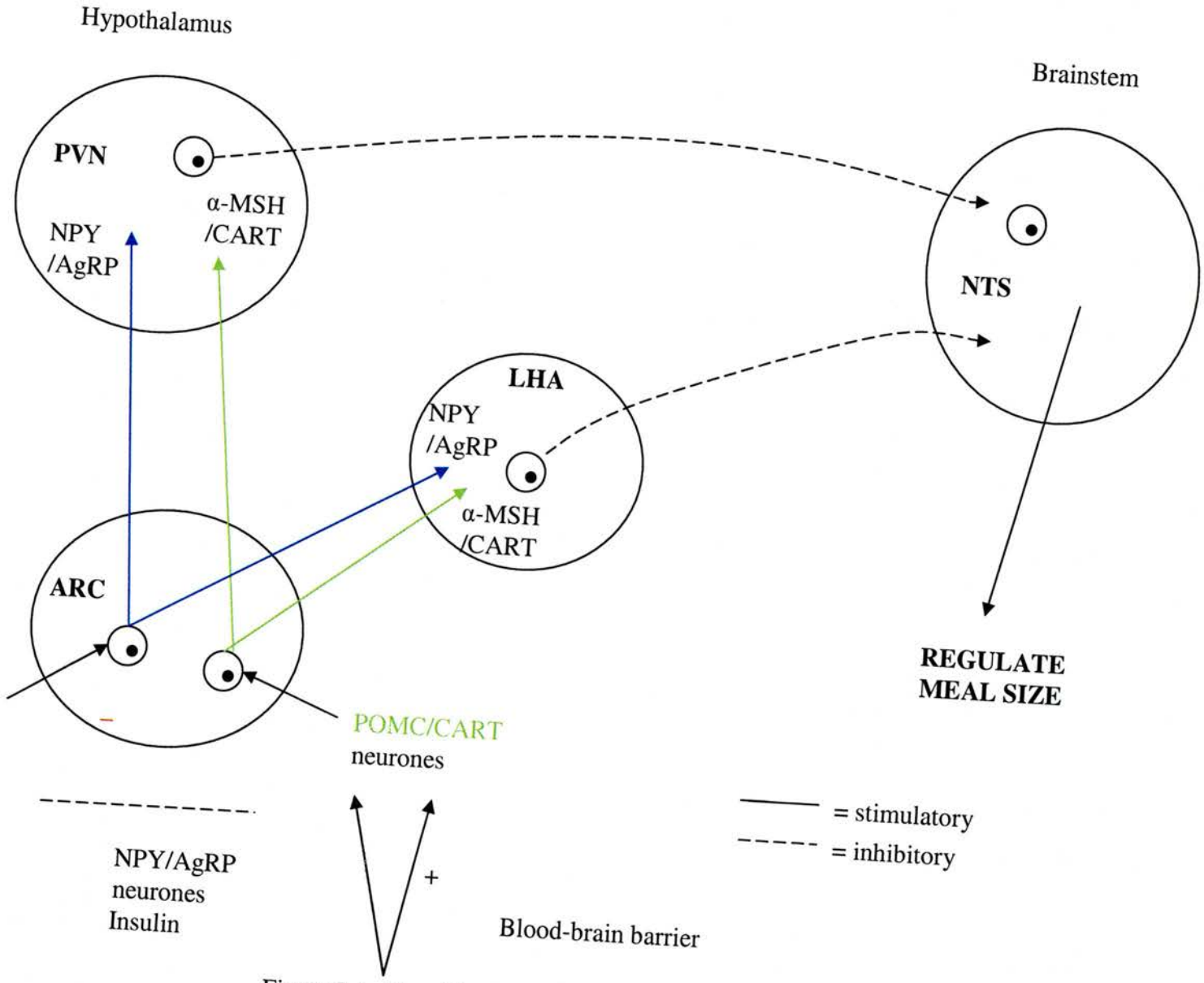


Figure 3.1: The effects of insulin in the central nervous system..
Insulin acts to inhibit NPY/AgRP neurones and stimulate POMC/CART neurones.

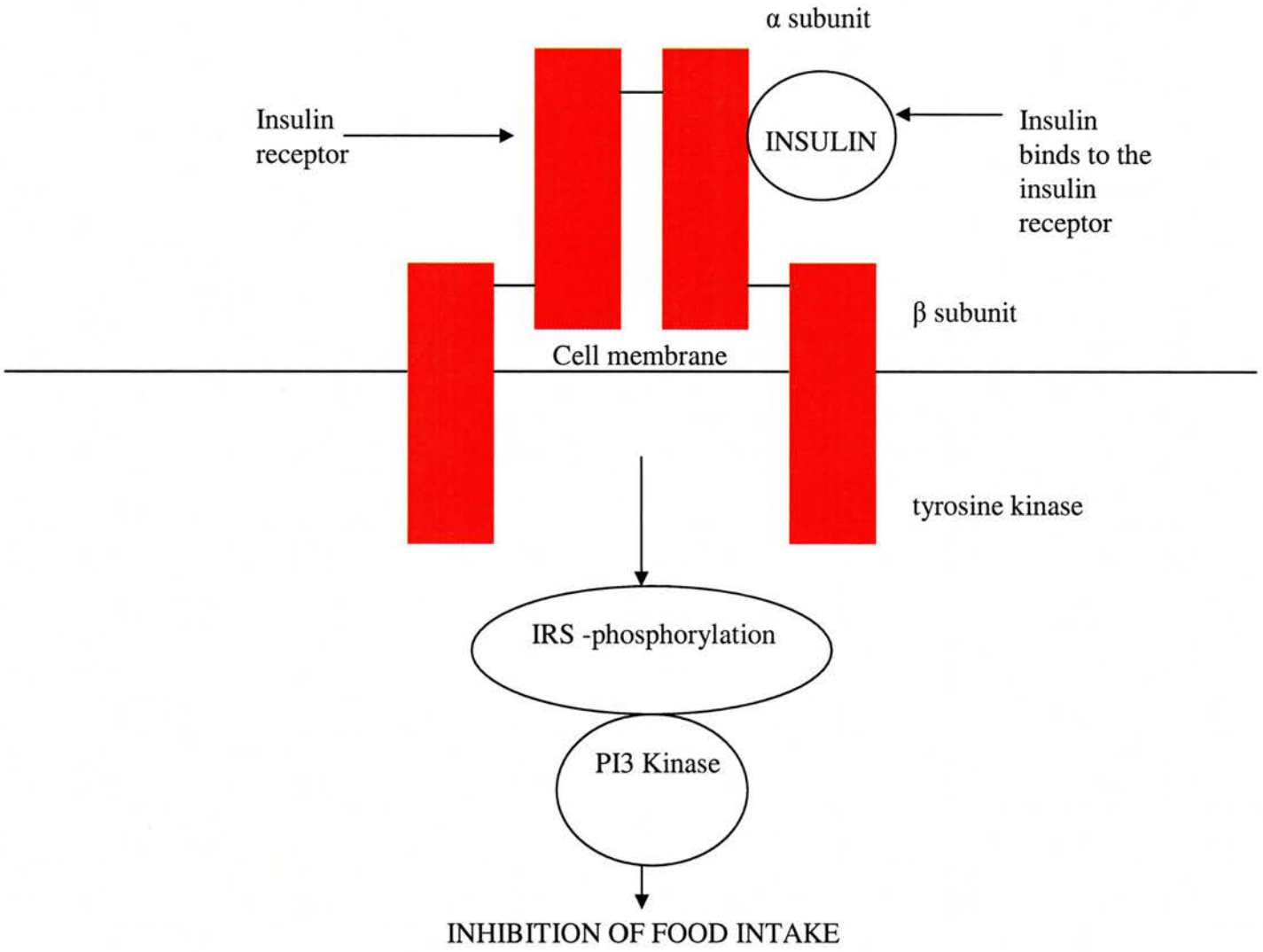


Figure 3.2: The activation of PI3-kinase following binding of insulin to the insulin receptor in relation to the effects on food intake.
IRS = insulin receptor substrate; PI3 = phosphatidyl inositol 3

(IRS-2). The classical insulin receptor substrates, IRS-1 and IRS-2 have both been identified in neurones (Burks *et al*, 2000). Several events follow activation of IRS-1. One potential intracellular signalling mechanism involves phosphatidylinositol 3 kinase (PI-3K). The activation of PI-3K leads to the inhibition of food intake (Niswender *et al*, 2001) (Figure 3.2).

3.1.3. Insulin entry into the CNS

Increased peripheral insulin level decreases feeding behaviour (Woods *et al*, 1985). The fact that this occurs, despite the endothelial tight junctions comprising the blood brain barrier which limits diffusion of peptides of this size, suggests that circulating insulin acts via a circumventricular organ or may enter the brain via a facilitated transport process (Pardridge *et al*, 1987). *In vitro* studies have shown that insulin transport across the blood brain barrier is dependent on its binding to insulin receptors (Bar *et al*, 1985). This led to the hypothesis that receptor-mediated insulin transport may facilitate the delivery of peripheral insulin to target tissues *in vivo* (Frank *et al*, 1983).

3.1.4 Insulin regulation of glucose production

There is interest in the role of insulin signalling in the central nervous system because of its peripheral role in plasma glucose regulation. It was shown that hepatic glucose production declines sharply during infusion of insulin into the third ventricle (Obici *et al*, 2002) and that this effect occurs independently of any change in circulating levels of insulin. This effect on blood glucose appears to be dependent on neuronal signal transduction between the insulin receptor substrate (IRS)-phosphatidylinositol 3-OH kinase (PI3K) pathway, because intracerebroventricular

(i.c.v.) injection of a PI3K inhibitor exerted the opposite effect (Obici *et al*, 2002). Hypothalamic insulin signalling is also required for the normal control of glucose production by the liver, as indicated by effects of insulin specific antibodies or antisense oligonucleotides directed against the insulin receptor injected into the third ventricle (Obici *et al*, 2002). Because of this it is thought that insulin action in the brain is a physiological determinant of glucose metabolism. An overview of insulin signalling controlling glucose homeostasis is shown in Fig.3.3.

Insulin causes blood glucose to enter cells, except in the brain, as it is needed, thereby decreasing blood glucose; consequent inhibition of insulin and stimulation of glucocorticoid secretion can counteract this action by mobilising glucose stores to make them freely available for the body undergoing a stress response. Hence, it is of interest to consider the effects of IIH on activation of the HPA axis.

3.1.5 Insulin-induced hypoglycaemia and activation of the HPA axis

IIH has been associated with activation of the HPA axis for many years (Gershberg *et al,* 1948). Insulin injection is followed by release of ACTH, β-endorphin, growth hormone (GH) and prolactin (PRL). The ACTH response appears to be dose related (Keller-Wood *et al*, 1981), but the nature of the pathways mediating this response is controversial. It was thought that ACTH secretion induced by IIH was mediated within the medial basal hypothalamus (MBH) (Aizawa *et al*, 1981). It was also *identified that lateral hypothalamic and hepatic portal glucose sensitive cells may well be responsible for mediating the increase in ACTH secretion (Shimizu *et al*, 1983).

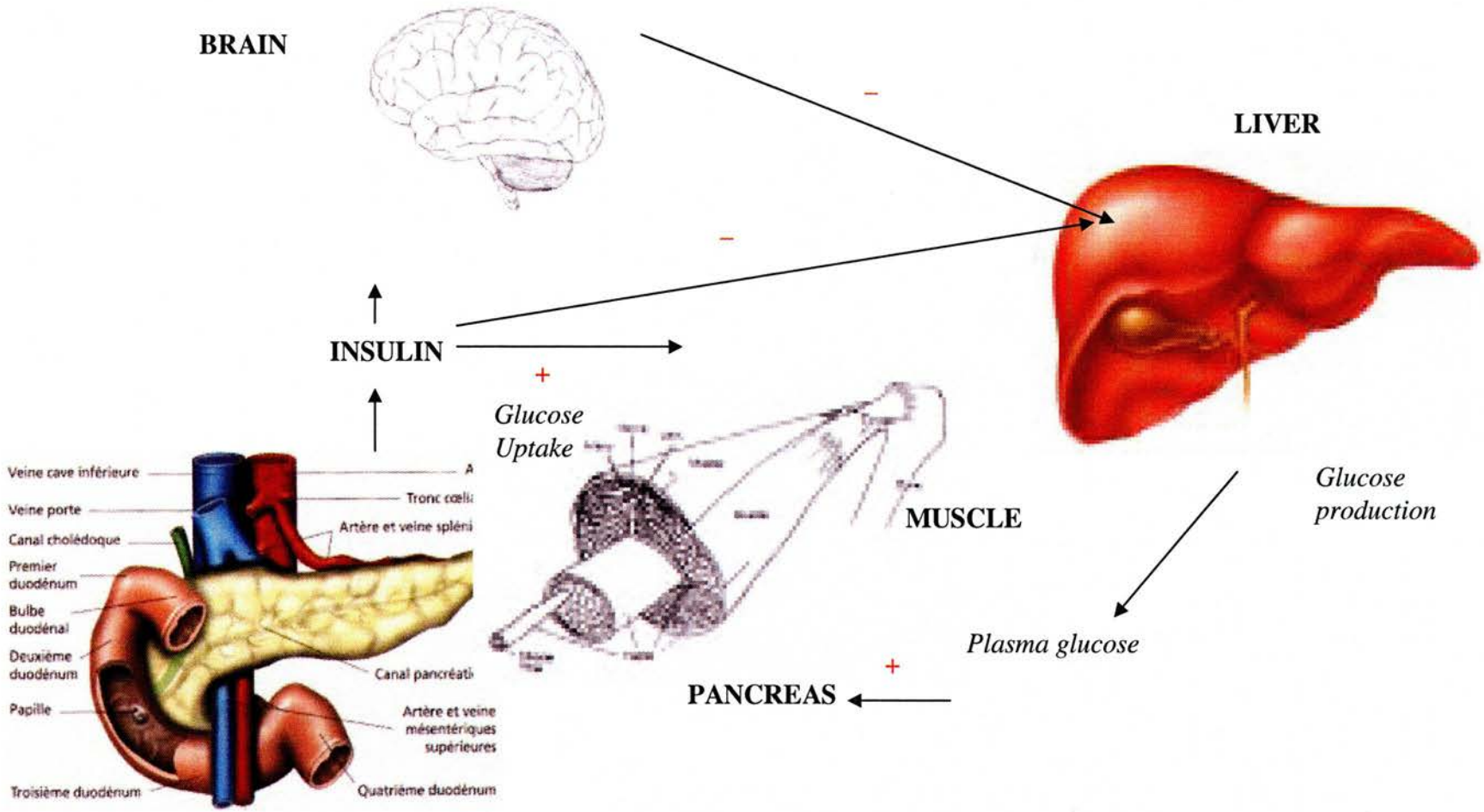


Figure 3.3: Central and peripheral insulin signals controlling glucose homeostasis
Pictures acquired from: pancreas www.diabetenet.com/le-pancreas.htm; muscle www.training.seer.cancer.gov/module_anatomy/unit4... ; liver www.digilander.libero.it/.../kapil/moremedi.htm ; brain www.cs.princeton.edu/gfx/proj/supcon/models/

ACTH secretion in response to IIH mediated within the basal hypothalamus

The primary site of action of insulin-induced-hypoglycaemia to stimulate ACTH secretion was investigated in rats with lesions of the MBH (Aizawa *et al*, 1981). Hypoglycaemia failed to provoke ACTH secretion in rats with lesions of the MBH. The data indicated that hypoglycaemia stimulates ACTH secretion through a direct effect in the MBH and not in the extrahypothalamic CNS or the anterior pituitary (Aizawa *et al*, 1981). Another study showed that the release of ACTH and corticosterone during IIH (Jezova *et al*, 1987) was markedly reduced in rats with lesions of the MBH. MBH lesions however resulted in normal circulating adrenaline and noradrenaline concentrations during IIH (Jezova *et al*, 1987).

ACTH secretion in response to IIH mediated within the LHA

The LHA is very important in overall regulation of feeding behaviour and body weight (Bernardis *et al*, 1996). It is crucial for the intense hunger that is caused by hypoglycaemia. Eating is one component of the metabolic responses that are mounted to restore normal blood glucose levels. Lesions of the LHA abolish the hyperphagic response to hypoglycaemia induced by insulin (Epstein *et al*, 1967). The LHA contains glucose-sensitive neurones, which are stimulated by IIH, and these neurones account for around 25% of the LHA neurones (Oomura *et al*, 1974). Hypoglycaemia activates these glucose sensitive neurones in part indirectly (Orsini *et al*, 1992) and pathways ascending from the brain stem are thought to be particularly important. These include a projection from the NTS (Ricardo *et al*, 1978), which relays information from vagal afferents including from glucoreceptors in the gut and liver (Paton *et al*, 1999).

It has been shown that the LHA neurones which respond to hypoglycaemia express the orexin peptides (Moriguchi *et al*, 1999). In this experiment it was shown that there was activation of orexin neurones in rats that were fasted and not allowed access to food but in those rats that were fasted and then given food there was no activation of orexin neurones (Cai *et al*, 2001).

3.1.6 Glucose sensing neurones

The brain regulates energy homeostasis by balancing energy intake, expenditure and storage. It has specialised neurones that receives neural and metabolic signals conveying information about the energy status of the body. The ability to sense and regulate glucose metabolism is critical because glucose is essential for neural function. There are two types of glucose sensing neurones, those that either increase glucose responsive or decrease glucose sensitive their firing rate as glucose levels rise (Oomura *et al*, 1974). Glucose-sensing neurones have been identified in the hypothalamus and brainstem, in particular in the LHA, VMH and the NTS (Anand *et al*, 1964). The increase in firing rate of glucose responsive neurones when glucose levels rise (Oomura *et al*, 1974), is a response modulated by a K^+ channel that is sensitive to the intracellular ratio of ATP to ADP called the ATP-sensitive K^+ channel (Trapp *et al*, 1997). Less is known of the mechanism(s) decreasing the firing of GS neurones when glucose levels rise. It has been proposed that GS activity is regulated by the Na^+-K^+-ATP pump (Oomura *et al*, 1974).

3.1.7. Aims of experiments

CRH and AVP are considered to be the principal stimulators of ACTH release after stress. Unclear, however, is the extent to which either CRH or AVP plays a role in this response. There have been several studies investigating IIH effects on activation of the HPA-axis in the rat (Plotsky *et al*, 1985). Here, in anaesthetised rats, hypothalamo-hypophysial portal blood concentrations of AVP were increased nearly 2-fold while CRH remained unchanged by IIH. IIH has also been associated with elevated levels of AVP in peripheral plasma in humans (Baylis *et al*, 1975). More recently CRH mRNA expression in the pPVN has been shown to increase in response to IIH (Suda *et al*, 1988). It has also been shown that there are increased levels of CRH in hypophysial portal blood in response to IIH in the rat (Guillaume *et al*, 1989). It has been suggested that the role of AVP in the rat becomes more predominant with severe hypoglycaemia (Caraty *et al*, 1990). All of these studies are complicated by whether anaesthetised animals were used and were fasted, and which route of administration was used. The different protocols used are summarised in table 3.1. In the present experiment the effects of IIH on the HPA axis were assessed in freely moving, unanaesthetised, unfasted rats.

It has also previously been shown that during late pregnancy the responsiveness of the HPA axis to physical and emotional stressors (Neumann *et al*, 1988) and metabolic signals is reduced (Brunton *et al*, 2003; 2006). This reduced responsiveness is evidently due primarily to reduced responsiveness of the parvocellular PVN CRH neurones. We investigated if the responsiveness of the HPA axis to IIH in late pregnancy provides a further example of reduced responses to a signal about metabolic status. IIH is complicated in pregnancy due to a resistance to

Authors	Anaesthetised	Fasted	Dose	Effect on HPA axis
Gazola *et al*, 2006	No	Yes	1IU/kg i.p.	no HPA axis effects
Han *et al*, 2005	No	No	6IU/kg i.p.	increased AMPK activation in the PVN
Chan *et al,* 2005	No	No	2.5IU/kg/day i.p. infusion	increased CRH mRNA and plasma ACTH
Koenig *et al,* 2005	No	No	2IU/kg i.p.	increased plasma corticosterone
Li *et al*, 2003	No	Yes	0.6IU/kg i.v.	increased CRH mRNA in the PVN
Umegaki *et al,* 2003	No	No	6IU/kg i.v.	no significant increase in ACTH to IIH
Paulmyer-Lacroix *et al,* 2002	No	No	2.5IU/kg i.p.	increased ACTH and CRH mRNA
Kawaguchi *et al,* 2002	No	No	1IU/kg i.v.	increased luteinising hormone
Evans *et al,* 2002	No	No	0.5IU/kg i.v. infusion	increased Fos in the PVN, DMH and ARC
Flanagan *et al,*2002	No	No	10IU/kg i.p. every day	increased adrenaline secretion
Cai *et al,* 2001	No	Yes	50IU/kg i.p.	increased plasma ACTH concentration
Osako *et al,* 1999	No	Yes	4IU/kg i.p.	increased CINK expression in the PVN
Romero *et al,* 1993	No	No	3IU/kg i.v.	increased plasma ACTH and corticosterone
Kjaer *et al,* 1993	No	Yes	3IU/kg i.p.	increased plasma ACTH and β-endorphin
Muret *et al,* 1992	No	Yes	3IU.kg i.p.	increased plasma ACTH and corticosterone
Goeij *et al*, 1992	No	Yes	3IU/kg i.p.	increased AVP but not CRH in ME
Grino *et al,* 1992	No	Yes	2.5IU/kg i.p.	increased plasma ACTH
Robinson *et al,*1992	No	Yes	4IU/kg i.v.	increased POMC and AVP but not CRH mRNA in the pPVN
Berkenbosch *et al,*1989	No	Yes	3IU/kg i.v.	increased CRH and AVP in ME
Guillaume *et al,* 1989	Yes	No	2.5IU/kg i.v.	increased ACTH and CRH
Suda *et al,* 1988	Yes	Yes	1IU/kg i.v.	increased CRH mRNA
Tozawa *et al,* 1988	No	No	10U/kg i.p and 2 IU/kg i.v	increased POMC mRNA in pituitary
Jezova *et al,* 1987	No	Yes	2.5IU/kg i.v.	increased plasma ACTH and corticosterone
Plotsky *et al,* 1985	Yes	Yes	0.3IU/kg i.v.	increased plasma ACTH and hypophysial portal AVP
Aizawa *et al,* 1981	No	No	5IU/kg i.p.	increased plasma ACTH
Baylis *et al,* 1980	No	No	3IU/kg i.p.	increased plasma AVP

Table 3.1: Protocols of insulin induced hypoglycaemia for studies on the HPA axis in the rat

CINK = cytokine-induced neutrophil chemoattractant
ME = median eminence

insulin's ability to promote glucose metabolism (Buchanan *et al*, 1990) and reduced β-cell responses to glucose (Costrini *et al*, 1971). This resistance is in part compensated for by the appearance of pancreatic insulin hypersecretion.

The first aim of the experiment was to investigate if IIH activates the HPA axis in unfasted, conscious virgin female rats; the second aim being whether this activation was altered in pregnant rats.

3.2 Methods

3.2.1 Animals

Female Sprague Dawley rats were used and housed individually after surgery. Rats were maintained as described in section 2.1.

3.2.2 Surgery

Rats were implanted with a jugular vein cannula under halothane anaesthesia. Surgery was performed under the conditions described in section 2.3.

3.2.3 Experiment 1 – The effect of IIH on the HPA axis – Insulin at 2U/kg

On the day of the experiment (day 21 of pregnancy) the cannulae were connected between 07:30-08:30h. The jugular vein cannula was attached to PVC extension tubing led out of the cage and connected to a 1ml syringe filled with heparinised saline (0.9% saline, 50 units/ml). Rats were left undisturbed for 90 minutes and a basal blood sample was taken. Thirty minutes after the first basal blood sample another sample to measure blood glucose was taken (<100μl) and rats were given either insulin (fast acting Actrapid; Novo Nordisk UK, 2U/kg dose, volume 0.5ml/kg) or sterile saline, (0.9%, 0.5ml/kg). Further blood samples (<100μl) were taken for glucose measurement at 10, 20, 30, 40, 50, 60, 90, 120, 150, 180 and 210 minutes after i.v. infusion. Glucose measurements were taken throughout the experiment using a Roche Accuchek Active Meter. Blood samples at 10, 30, 60 and 120 minutes (0.55ml) were placed into eppendorf tubes containing 50μl of chilled 5% EDTA. After each blood sample, blood was replaced with 0.9% sterile saline. Plasma was separated by centrifugation and stored at -20°C until radioimmunoassay.

Insulin was given intravenously as this is a rapid way of achieving hypoglycaemia, and avoids possible interference with fetuses that may have occurred with intra-peritoneal administration. Rats were killed by conscious decapitation 4 hours after insulin infusion (this time point has previously been shown as the optimum time for showing increased CRH and AVP mRNA expression in the PVN following stress) (Harbuz *et al*, 1989). Rats were examined post-mortem to check the number of fetuses.

3.2.4. Experiment 2 – Effect of IIH on the HPA axis – Insulin at 10U/kg

On the day of the experiment (day 21 of pregnancy) the cannulae were connected between 07:30-08:30h. The jugular vein cannula was attached to PVC extension tubing led out of the cage and connected to a 1ml syringe filled with heparinised saline (0.9% saline, 50 units/ml). Rats were left undisturbed for 90 minutes and a basal blood sample was taken. 30 minutes after the first basal blood sample another basal blood sample was taken and rats were given either insulin (fast acting Actrapid, 10U/kg dose volume 0.5ml/kg) or sterile saline, 0.9% (0.5ml/kg). Blood samples were taken at 15, 30, 60, and 120 minutes after i.v. infusion. Blood samples (0.55ml) were placed into eppendorfs containing 50µl of chilled 5% EDTA. After each blood sample, blood was replaced with 0.9% sterile saline. Glucose measurements were taken as described above. Plasma was separated by centrifugation and stored at -20°C until radioimmunoassay. Rats were killed by conscious decapitation 4 hours after insulin as described above.

3.2.5 *In Situ* Hybridisation

Brains were coronally sectioned at 15μm and sections containing the pPVN were mounted onto Polysine slides (BDH) (see section 2.4.2). To measure CRH mRNA expression a 42-mer oligonucleotide probe (MWG-Biotech) (5'-CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GCC- 3') was used complementary to bases 496-537, which encode amino acids 22-35 of the rat CRH peptide (Jingami *et al*, 1985).

To measure AVP mRNA expression a 36-mer oligonucleotide probe (MWG-Biotech) (5'-GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT-3') was used complementary to bases 486-521 of rat AVP mRNA (Majzoub *et al*, 1983). Both probes were labeled with ^{35}S as described in section 2.4.2. The melting temperature for the CRH mRNA probe was 78°C and for the AVP mRNA probe was 76°C, therefore the heated SSC washes were performed at 58°C and 56°C respectively. Once dry the sections were dipped in photographic emulsion under safelight conditions and stored at 4°C. The exposure time was 10 weeks for CRH mRNA and 7 days for AVP mRNA. The number of positive cells for CRH mRNA and the grain density over the pPVN for AVP mRNA were measured with the image analysis system described in 2.4.2.

3.2.6 ACTH Radioimmunoassay

Plasma ACTH concentration was determined using a commercially available kit as described in section 2.7.3. The sensitivity of the ACTH assay was 8pg/ml. The intra-assay variation was <9%. All samples from an experiment were measured in the same assay.

3.2.7 Statistics

A two-way analysis of variance (ANOVA) was used to analyse differences between groups. For blood sampling experiments a two-way ANOVA with repeated measures was used. If ANOVA showed significant effects Student Newman Keuls multiple comparison tests were used to locate differences between groups. A p value of less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 Effect of Insulin (2U/kg) on the HPA axis

Blood Glucose Concentration

Analysis of plasma glucose concentration using two-way RM ANOVA showed a statistically significant difference in blood glucose concentration among the virgin and pregnant groups ($p<0.001$). Basal blood glucose concentrations were significantly lower in pregnant rats than virgins ($p<0.05$; two way RM ANOVA). I.v. injection of insulin significantly reduced blood glucose in the virgin group ($p<0.001$; two-way RM ANOVA) within 10 min (2.3 ± 0.03 vs 5.2 ± 0.47 mmol/l) with glucose concentrations returning to basal by 90 min (Fig.3.4). I.v. injection of insulin significantly reduced blood glucose in the pregnant group ($p<0.001$; two-way RM ANOVA) within 10 min (2.1 ± 0.10 vs 4.0 ± 0.23 mmol/l) with glucose concentrations returning to basal by 90 min (Fig.3.4). There were no significant differences in blood glucose concentration following i.v. saline in either the virgin or the pregnant group (Fig.3.4).

Plasma ACTH concentration

Analysis of plasma ACTH concentration using a two-way RM ANOVA showed there was not a statistically significant difference in basal plasma ACTH concentrations among the virgin and pregnant groups (Fig 3.5). I.v. injection of insulin or saline had no significant effect on ACTH secretion in either the virgin or the pregnant group (Fig.3.5 and Fig.3.6).

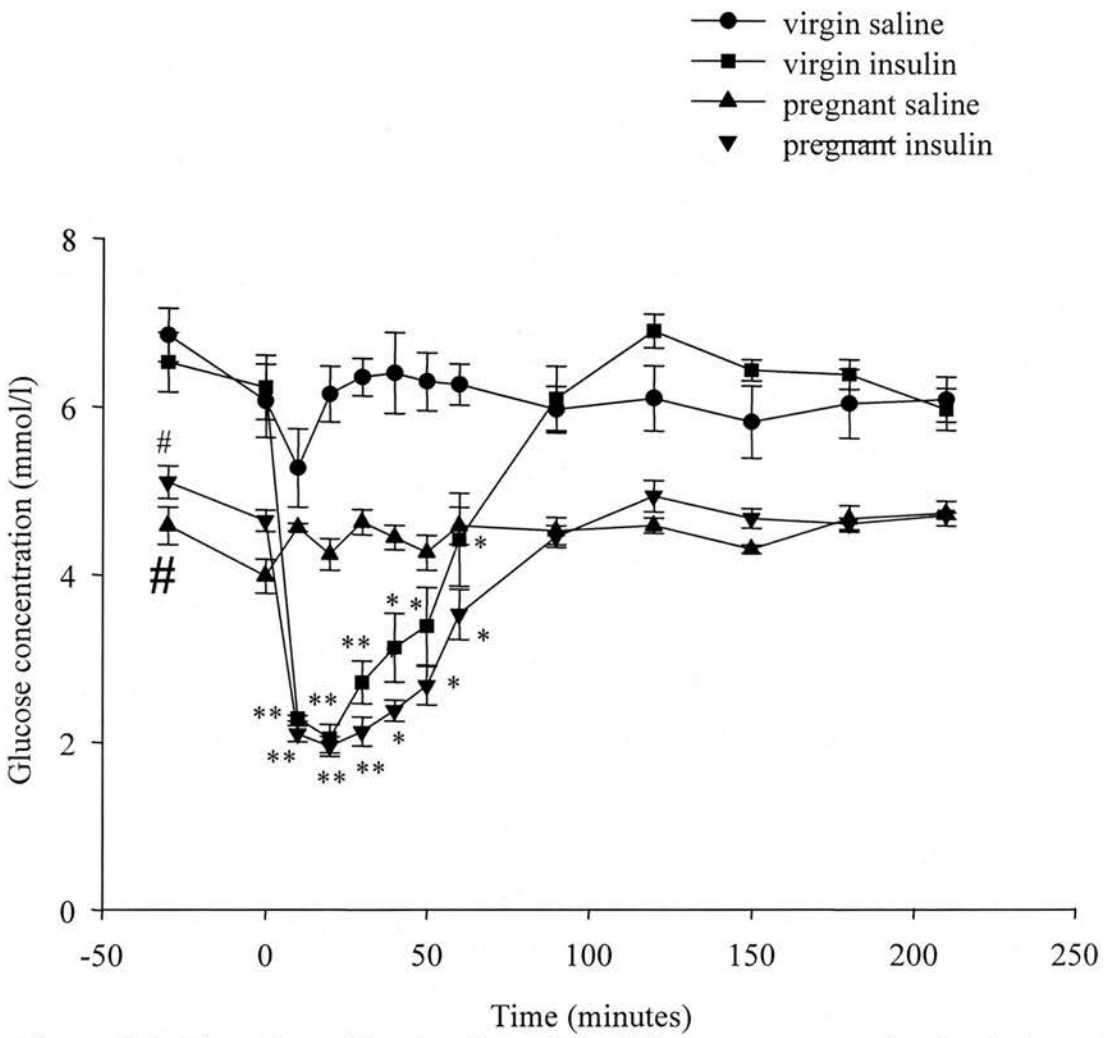


Figure 3.4: The effect of i.v. insulin on blood glucose concenration in virgin and pregnant rats. A basal blood sample was collected before i.v. administration of Insulin (2U/kg). Further blood samples were withdrawn -10, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180 and 210 minutes post-injection. Values are group means ±SEM. Virgin/saline, n-=6; virgin/insulin,n=7; pregnant/saline, n=5; pregnant/insulin, n=5. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests:
*p<0.05, **p<0.001 significantly different from basal
#p<0.05 vs virgin

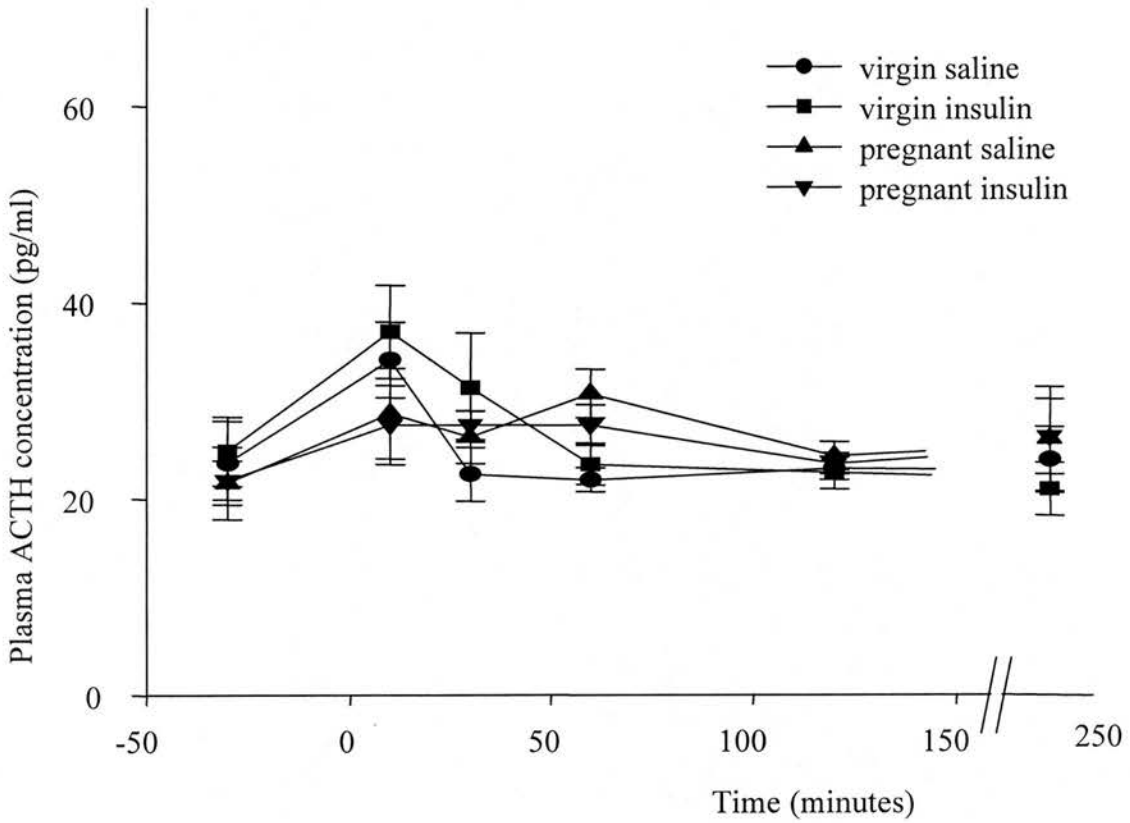


Figure 3.5: The effect of i.v. insulin on plasma ACTH concentration in virgin and pregnant rats. A basal blood samples was taken 30 minutes before i.v. administration of insulin (2U/kg). Further blood samples were Taken 10, 30, 60, 120 and 250 (trunk blood) minutes post-injection. Values are group means ± SEM. Virgin/saline, n=6; virgin/insulin, n=7; Pregnant/saline, n=5; pregnant/insulin, n=5. Two-way ANOVA for repeated measures.

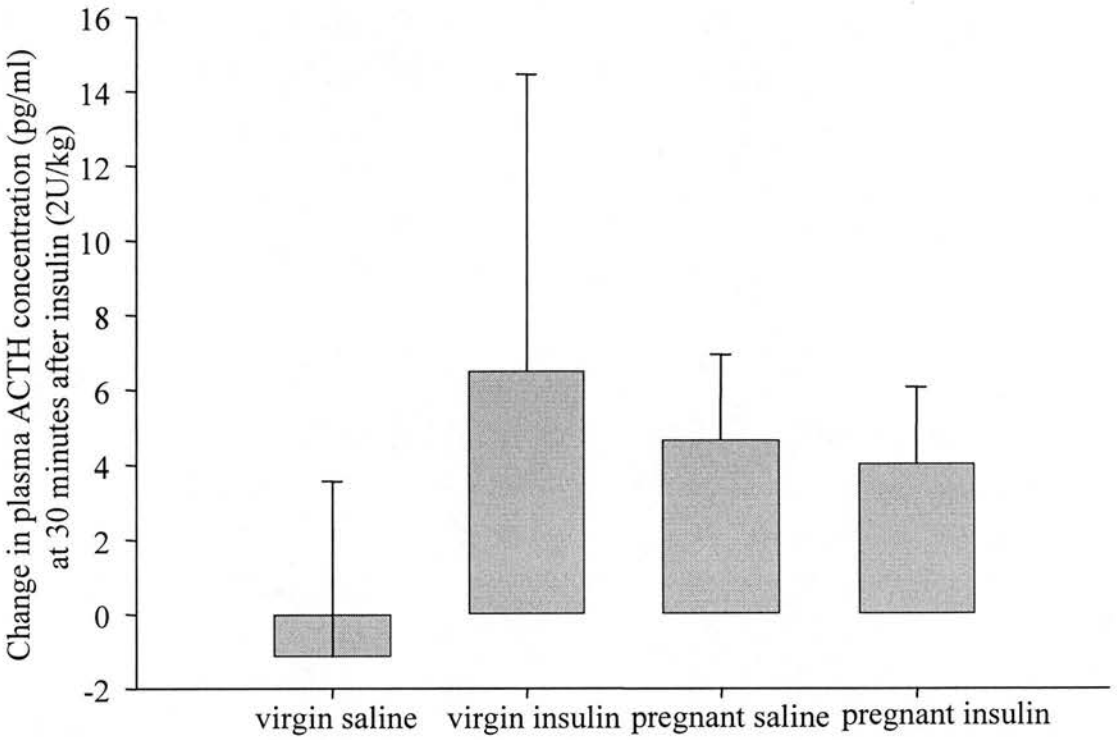


Figure 3.6: The effect of i.v. insulin on plasma ACTH concentration in virgin and pregnant rats. Delta values for the measurements at 30 minutes after insulin (2U/kg) injection subtracted from the basal blood sample were calculated. Values are the group means ± SEM. Virgin/saline, n=6; virgin/insulin, n=7; pregnant saline, n=5; pregnant/insulin, n=5. Two-way ANOVA.

3.3.2 Effect of Insulin (10U/kg) on the HPA axis

Blood glucose concentration

The dose of insulin was increased to 10U/kg since 2U/kg did not increase ACTH secretion. Analysis of blood glucose concentration using two-way RM ANOVA showed a statistically significant difference in plasma glucose concentration among the virgin and pregnant groups ($p<0.001$). Basal blood glucose concentrations were significantly lower in pregnant rats than virgins ($p<0.05$; two way RM ANOVA). I.v. injection of insulin significantly reduced blood glucose in the virgin group ($p<0.001$; two-way RM ANOVA) within 15min (2.1 ± 0.14 vs 6.2 ± 0.44 mmol/l) with glucose concentrations returning to basal by 120 min (Fig.3.7). I.v. injection of insulin significantly reduced blood glucose in the pregnant group ($p<0.001$; two-way RM ANOVA) within 15 min (1.7 ± 0.18 vs 4.0 ± 0.23 mmol/l) with glucose concentrations returning to basal by 120 min (Fig.3.8). There were no significant differences in blood glucose concentration following i.v. saline in either the virgin or the pregnant group (Fig.3.7).

Plasma ACTH concentration

Basal plasma concentrations of ACTH were not different among groups. I.v. injection of insulin significantly increased ACTH secretion in the virgin group ($p=0.03$; two-way RM ANOVA) (Fig.3.8 and Fig. 3.9) within 15 min (103.3 ± 21.6 vs 65.9 ± 5.1 pg/ml) with plasma ACTH concentrations returning to basal by 120 min (Fig.3.8). I.v. injection of insulin significantly increased plasma ACTH concentration in the pregnant group ($p=0.04$; two-way RM ANOVA) within 15 min (79.5 ± 16.5 vs 44.4 ± 4.6 pg/ml) with plasma ACTH concentrations returning to

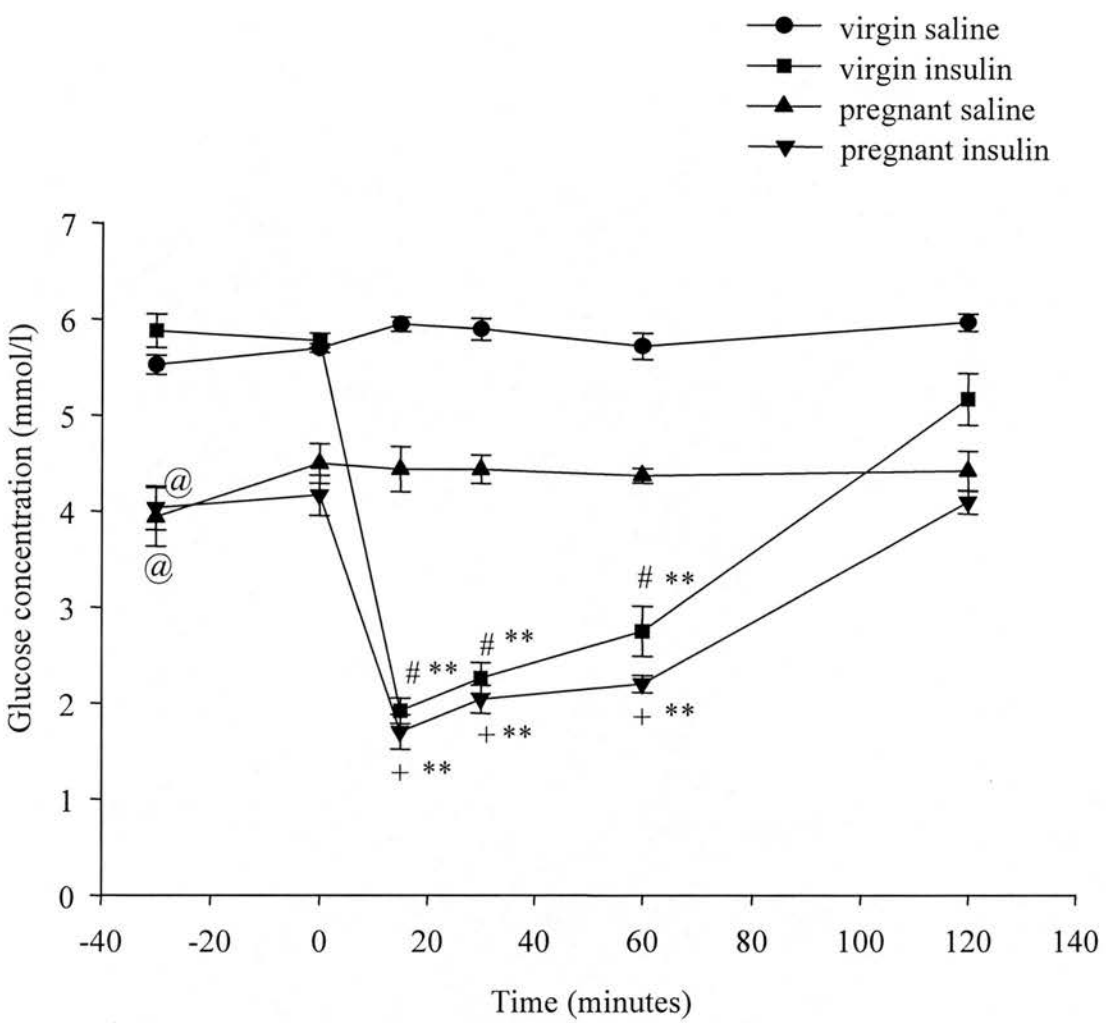


Figure 3.7:The effect of i.v. insulin on blood glucose concentration in virgin and pregnant rats. Basal blood samples were collected 1 and 30 minutes before i.v. administration of insulin (10U/kg). Further blood samples were withdrawn 15,30, 60 and 120 minutes post injection. Values are group means ± SEM. Virgin/saline, n=5; virgin/insulin, n=5; pregnant/saline, n=5; pregnant/insulin, n=6.
Two-way ANOVA for repeated measures followed by Student Newman-Keuls multiple comparison tests: **$p<0.001$, significantly different from basal, #$p<0.05$ significantly

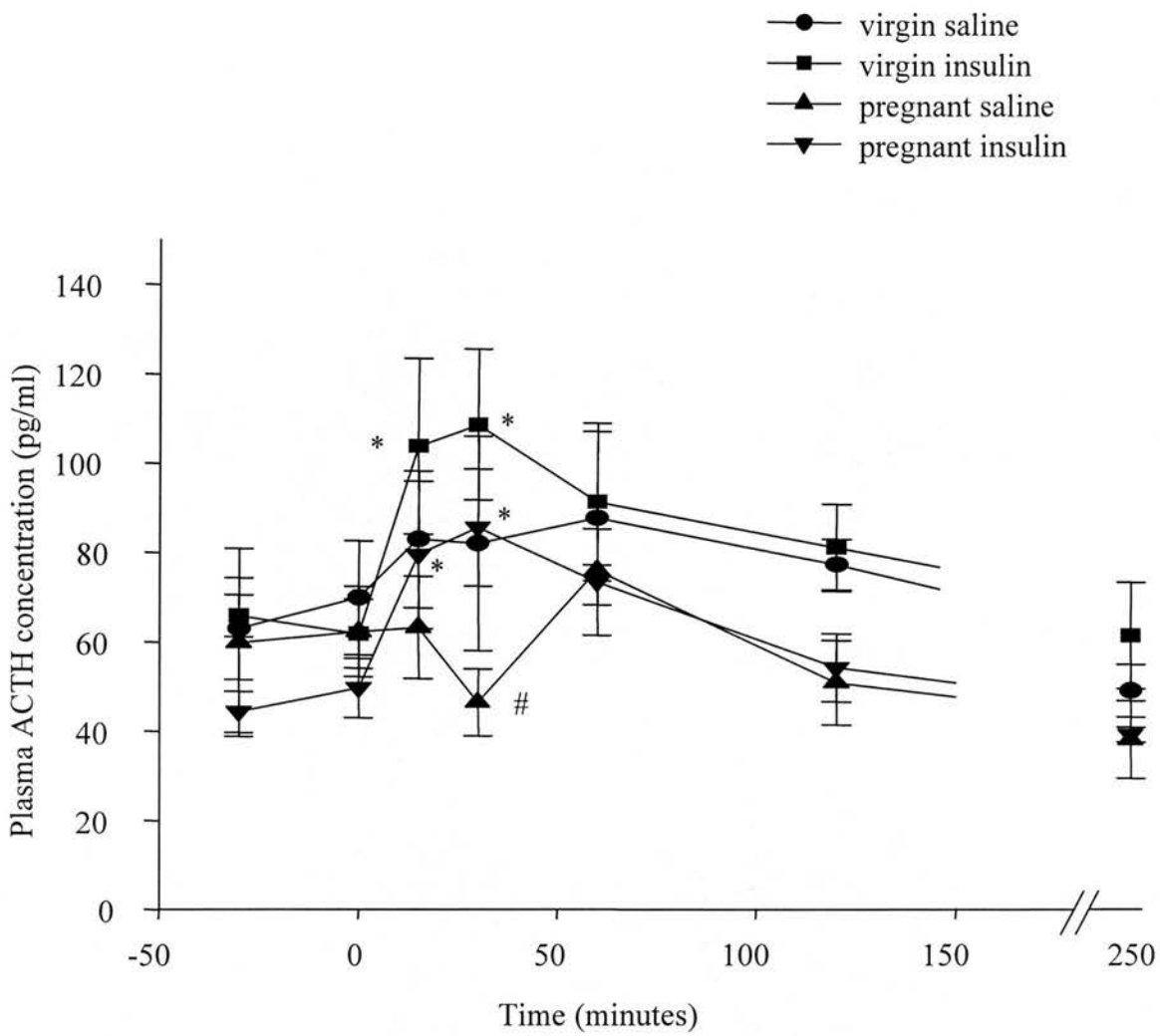


Figure 3.8: The effect f i.v. insulin on plasma ACTH concentration in virgin and pregnant rats. Two basal blood samples were taken 30 and 1 min prior to i.v. Insulin 10U/kg/rat. Further blood samples were taken 15, 30, 60, 120 and 240 (trunk blood) min post-infusion. Values are the group means ±SEM. Virgin/ saline, n=5; virgin/insulin, n=5; pregnant/saline, n=5' pregnant/insulin, n=6. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests. *$p<0.05$ significantly different from insulin groups.

basal by 120 min (Fig.3.8 and Fig.3.9). There were no significant differences in plasma ACTH concentration following i.v. saline in either the virgin or the pregnant group (Fig.3.8).

Parvocellular PVN (pPVN) CRH mRNA expression

Analysis of CRH mRNA expression in the pPVN using a two-way ANOVA showed there was not a statistically significant difference in positive cell counts for CRH mRNA expressing neurones between virgin and pregnant rats. I.v. insulin did not significantly increase CRH mRNA expression in the pPVN in either the virgin or the pregnant group 4 hours after insulin (10U/kg) (Fig.3.10 and Fig.3.11).

Parvocellular PVN (pPVN) AVP mRNA expression

Analysis of AVP mRNA expression using a two-way ANOVA showed there was not a statistically significant difference in grain density for AVP mRNA expressing neurons between virgin and pregnant rats. I.v. insulin significantly increased AVP mRNA expression in the pPVN in the virgin group 4 hours after insulin (10U/kg) ($p= 0.005$; 2 way ANOVA) (0.1 ± 0.03 vs control 0.03 ± 0.01 mm^2/mm^2) (Fig 3.12 and Fig 3.13). I.v. insulin significantly increased AVP mRNA expression in the pPVN in the pregnant group 4 hours after insulin (10U/kg) ($p=0.028$; 2 way ANOVA) (0.07 ± 0.02 vs control 0.03 ± 0.01 mm^2/mm^2) (Fig 3.12 and Fig 3.13). There was no significant difference in AVP mRNA expression after insulin between the virgin and the pregnant groups.

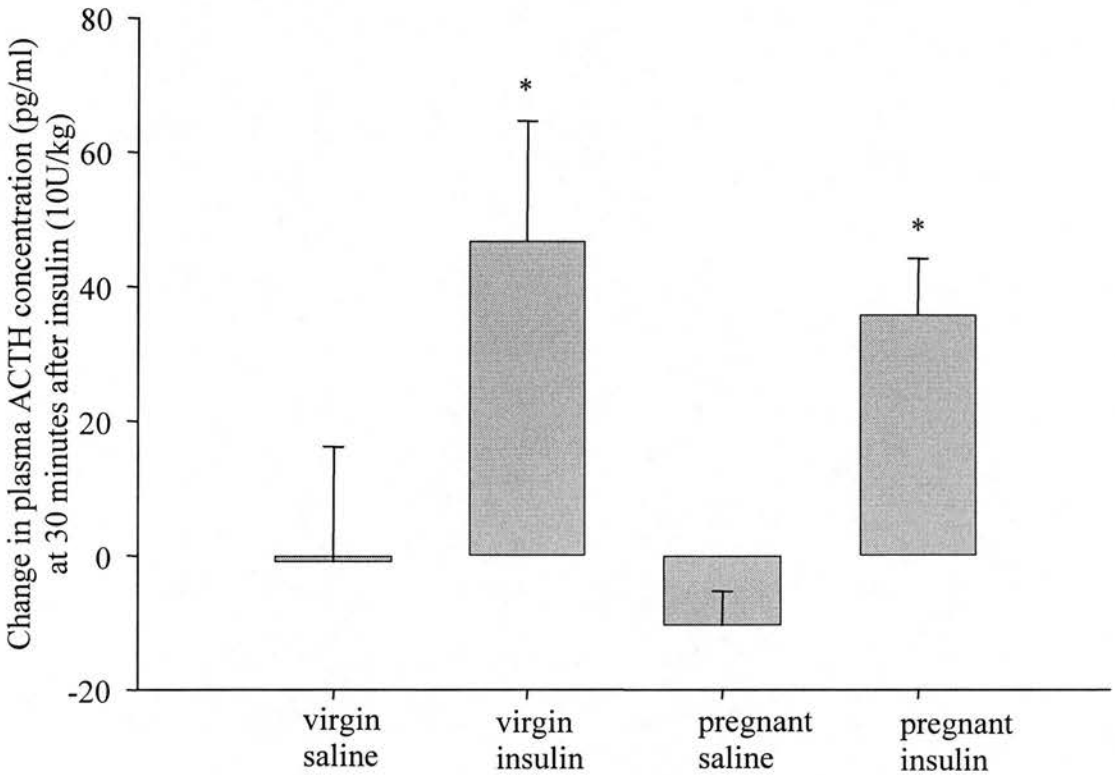


Figure 3.9: The effect of i.v. insulin on plasma ACTH concentration in virgin and pregnant rats. Delta values for the measurements at 30 minutes after insulin 10U/kg Subtracted from the first basal blood sample was calculated. Values are the group means ±SEM. Virgin/saline, n=5; virgin/insulin, n=5; pregnant/saline, n=5; pregnant/insulin, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test. *$p<0.05$ vs vehicle

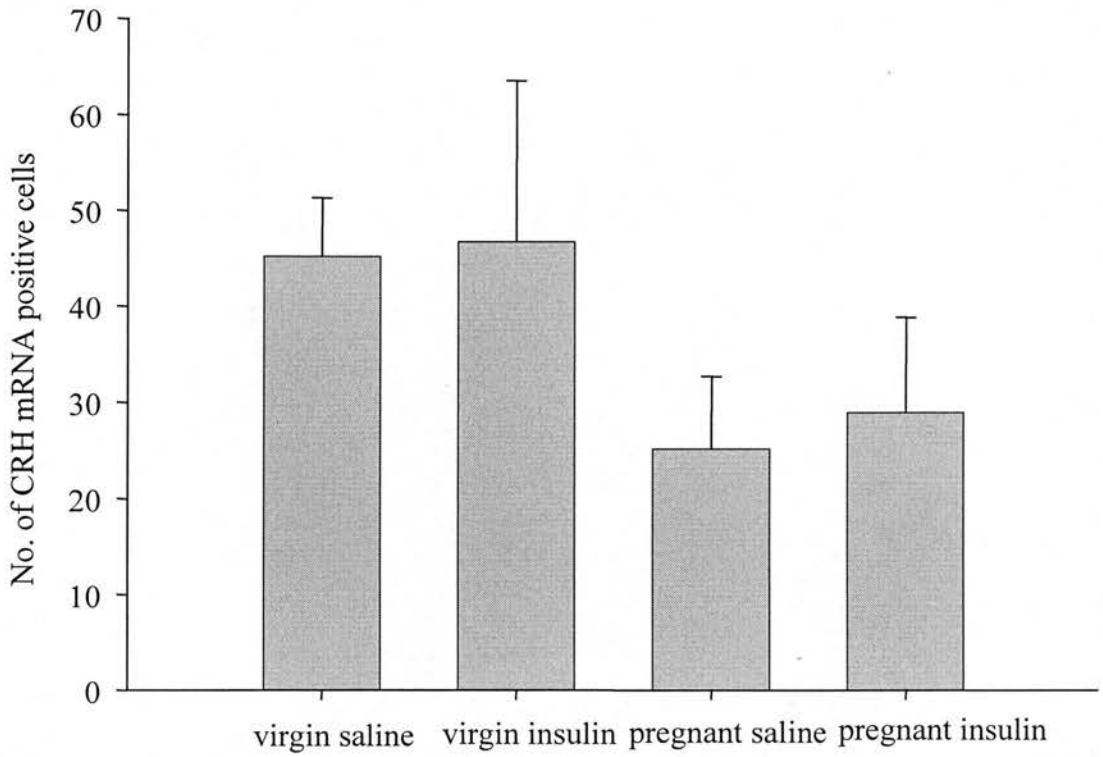


Figure 3.10: The effect of i.v. insulin on CRH mRNA in virgin and pregnant rats. The rats were killed by conscious decapitation 4 hours after insulin injection (10U/kg) and brains processed for CRH mRNA ISH on sections containing the PVN. CRH mRNA expressing neurones in the pPVN were counted.
Values are the group means ± SEM. Virgin/saline, n=5; virgin/insulin, n=5; pregnant/saline, n=5; pregnant/insulin, n=6. Two-way ANOVA.

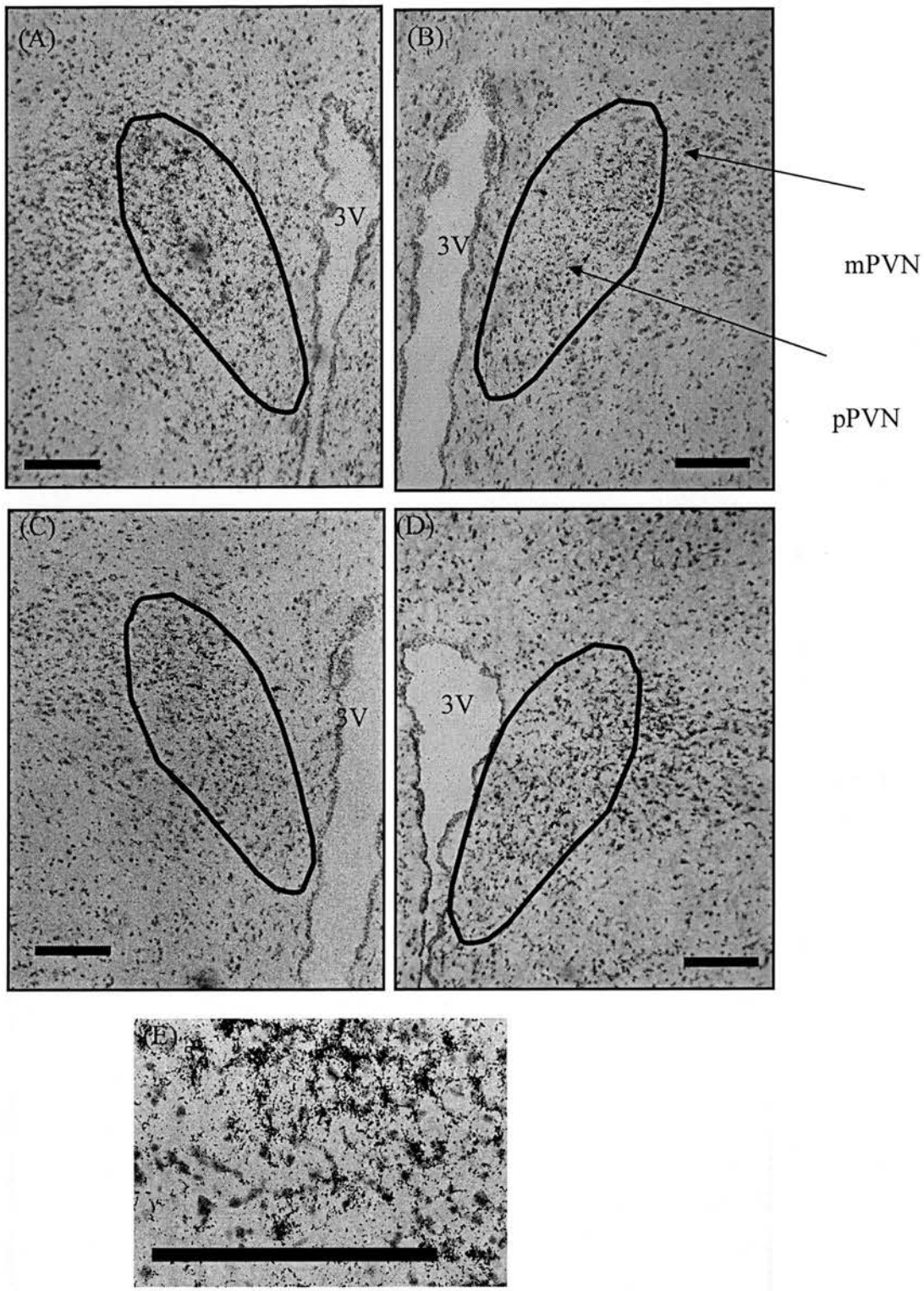


Figure 3.11: Representative photomicrographs of coronal sections through the PVN in virgin and pregnant rats. Sections were hybridised with a ^{35}S labelled probe complementary to CRH mRNA for (A) virgin saline; (B) virgin insulin; (C) pregnant saline; (D) pregnant insulin; (E) representation of positive cells at x 40 magnification. mPVN = magnocellular PVN; pPVN = parvocellular PVN. Scale bar: 100 μm.

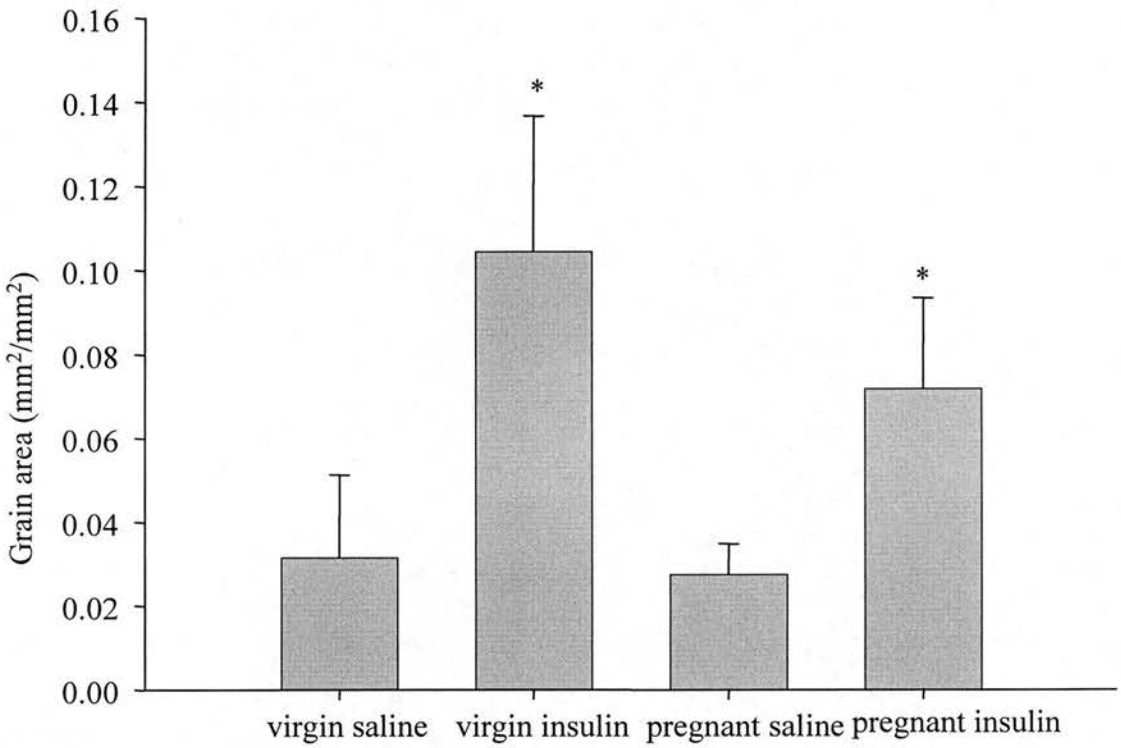


Figure 3.12: The effect of i.v. insulin on AVP mRNA in virgin and pregnant rats.
The rats were killed by conscious decapitation 4 hours after insulin injection 10U/kg and processed for AVP mRNA ISH on sections containing the pPVN. AVP mRNA expression in the pPVN was measured as grain per unit area.
Values are the group means ±SEM. Virgin/saline, n=5; virgin/insulin, n=5; Pregnant/saline, n=5; pregnant/insulin, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs saline.

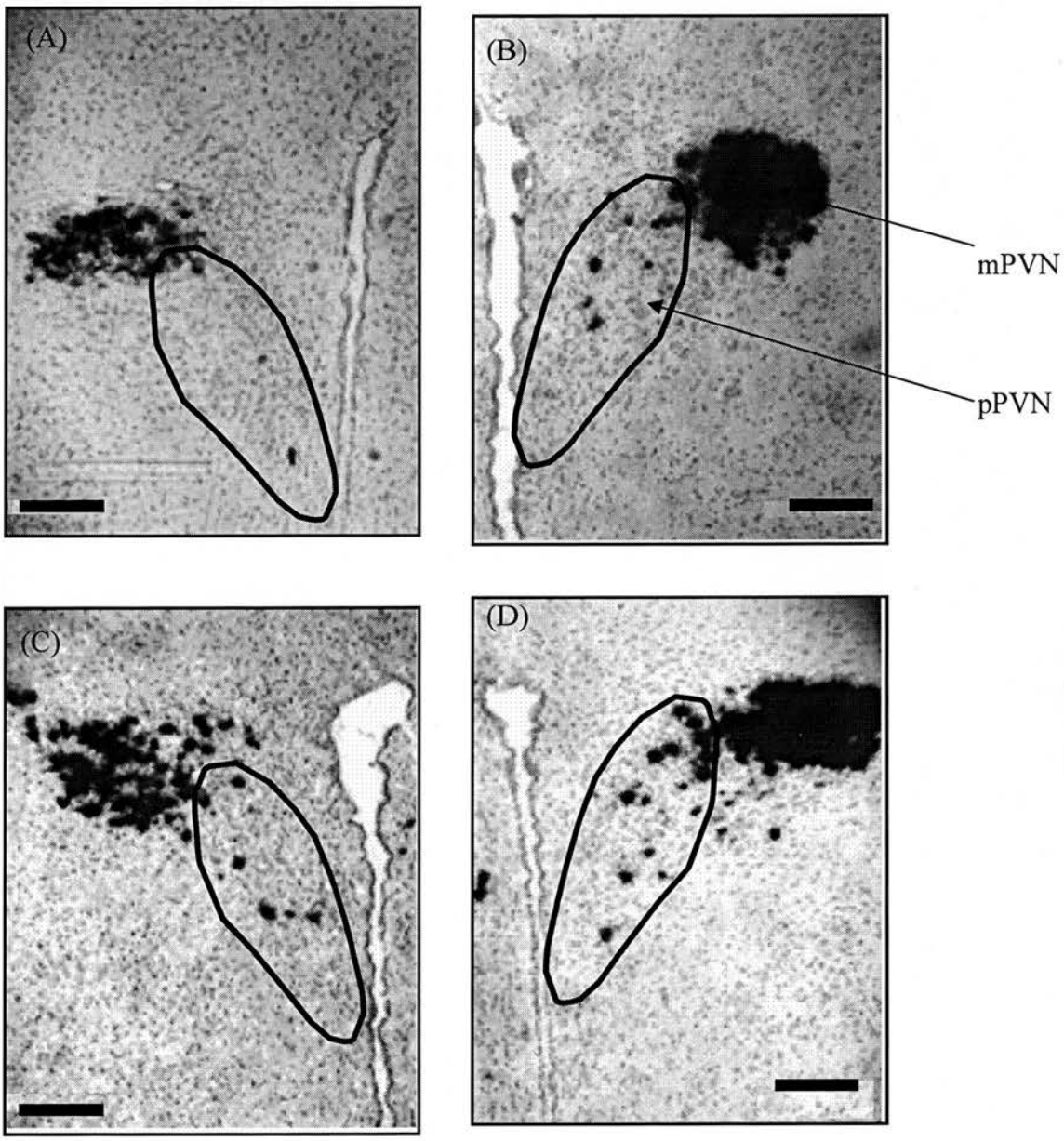


Figure 3.13: Representative photomicrographs of coronal sections through the PVN in virgin and pregnant rats. Sections were hybridised with a ^{35}S labelled probe complementary to AVP mRNA for (A) virgin saline; (B) virgin insulin; (C) pregnant saline; (D) pregnant insulin; Scale Bar: 100μm. mPVN = magnocellular PVN, pPVN = parvocellular PVN, 3V = 3rd ventricle

3.4 Discussion

3.4.1. IIH stimulation of ACTH secretion

We have shown that hypoglycaemia induced by i.v. insulin at 10U/kg stimulates ACTH secretion in female rats. This corresponds with previous studies in male rats (Plotsky *et al*, 1985, Jezvoa *et al* 1987). There was no effect seen on either ACTH or corticosterone secretion when insulin was given at a dose of 2U/kg in any group, despite a substantial decrease in blood glucose concentration. The higher dose of insulin decreased blood glucose concentration for longer than the lower dose.

3.4.2. The involvement of CRH and AVP in the stimulation of ACTH

CRH and AVP are considered to be the principal stimulators of ACTH release. The extent to which CRH and AVP are involved in the ACTH response to IIH is unclear. It has previously been shown that hypothalamo-hypophysial portal blood concentrations of AVP are increased following IIH with no changes in CRH (Plotsky *et al*, 1985). IIH has also been associated with increased levels of peripheral plasma AVP in humans, which reflects magnocellular AVP neurone activation (Baylis *et al*, 1980). Robinson *et al*, (1992) have shown that in response to IIH there is an increase in peripheral plasma AVP with no changes in PVN AVP mRNA expression. The study used PVN punches to determine AVP mRNA content. It is suggested that parvocellular neurones express less than 0.5% of the total AVP in the PVN (Robinson *et al,* 1992). Thus only a very marked increase in the levels of parvocellular AVP mRNA would have been detected with the punching technique. In this study we found that IIH increased pPVN AVP mRNA expression in both virgin

and pregnant rats. This corresponds with the increase in plasma ACTH seen in both virgin and pregnant rats.

Increases in levels of pPVN CRH mRNA (Suda *et al*, 1988) and ARC POMC mRNA (Tozawa *et al*, 1988) have been reported after IIH. The study by Suda *et al* (1988) was done using anaesthetised rats. Another study by Robinson *et al* (1992) in freely moving unanaesthetised rats showed that there was no change in the level of pPVN CRH mRNA 90 minutes after IIH. In these studies similar increases in ACTH (and corticosterone) plasma levels were achieved to the present study. Robinson *et al* (1992) showed that the duration of hypoglycaemia was longer than in the Suda *et al* (1988) study. In the present study we found no significant increase in pPVN CRH mRNA 4 hours after IIH in either the virgin or pregnant rats, suggesting that the stimulation of ACTH secretion by IIH does not involve CRH. It has previously been suggested that CRH may play a permissive role in activation of the HPA axis by IIH (Berkenbosch *et al*, 1989) or that IIH stimulates the release of CRH peptide (Guillaume *et al*, 1989) without causing alterations in the level of CRH mRNA. It would seem therefore that IIH may increase ACTH secretion by preferentially activating pPVN vasopressin-producing neurones. The mechanism whereby this is achieved requires further study. Importantly the mechanism is intact during late pregnancy.

AVP mRNA was measured in the parvocellular region of the PVN. It is very difficult however to distinguish between the parvocellular and magnocellular AVP neurones and some of the magnocellular neurones are mixed with the parvocellular neurones in the PVN. Basal AVP mRNA expression is much lower in the parvocellular region than the magnocellular region. It is thought anyway however that magnocellular

AVP neurones may contribute to HPA regulation (Landgraf *et al,* 2002) although magnocellular AVP neurones usually respond very slowly after stress (Ma *et al,* 1999).

3.4.3. Blood glucose concentration

Mammalian pregnancy near term is characterised by resistance to insulin's ability to promote glucose metabolism (Buchanan *et al*, 1990) as well as by augmented β-cell responses to glucose (Costrini *et al*, 1971). Experimental data from the rat have shown that insulin resistance during late pregnancy is due to an impairment of insulin's ability to stimulate glucose uptake in peripheral tissues and to suppress hepatic glucose production (Leturque *et al*, 1986). Insulin resistance in late pregnancy is in part compensated for by the appearance of pancreatic insulin hypersecretion (Rossi *et al*, 1993). Nonetheless, basal blood glucose concentrations were significantly lower in pregnant animals compared to virgins. This corresponds with previous studies which have shown that basal glucose levels decline as pregnancy progresses (Rossi *et al*, 1993). This could be due to the increasing metabolic demands of the fetuses on the mother at this time. Blood volume in the pregnant mother is also increased; this is perhaps another reason for the observed reduced basal blood glucose concentration (Longo 1983). Blood glucose concentrations were significantly reduced to the same level by IIH in both virgin and pregnant rats for the same time period with levels returning to basal concentrations by 60 minutes. Before beginning the present experiment, in pilot studies pregnant rats were given different doses of insulin as they have been reported to show a 25-30% reduction in the clearance of exogenous insulin (Rossi *et al*, 1993). However, as different doses produced equal degrees of hypoglycaemia in pregnant and virgin

animals it was decided to use the same concentration of insulin in both virgin and pregnant rats in the present experiment. This also meant that the rats were exposed to similar doses of insulin; this is important because the consequences of IIH may be due to insulin, or hypoglycaemia, or an interaction between these.

3.4.4. The effect of IIH on the HPA-axis in pregnancy

It has previously been shown that during late pregnancy the responsiveness of the HPA axis to physical and emotional stressors (Neumann *et al*, 1988; Brunton *et al,* 2005) and central neuropeptides signalling metabolic state is reduced (Brunton *et al*, 2003; 2006 see chapters 5 and 6). This reduced responsiveness is thought to be due primarily to reduced responsiveness of the parvocellular PVN CRH/AVP neurones or their inputs. In this study we have shown that IIH stimulates ACTH secretion and pPVN AVP mRNA expression similarly in both virgin and pregnant rats, yet we have shown that there is no stimulation of the pPVN CRH neurones, as assessed by measuring CRH mRNA expression, in response to IIH in either the virgin or late pregnant rats.

3.4.5 Conclusions

In summary, we have shown that IIH stimulates the hypothalamo-pituitary (HP) axis similarly in both virgin and late pregnant rats. Insulin lowered blood glucose concentration to a similar level in both virgin and pregnant rats. IIH did not increase pPVN CRH mRNA expression in either the virgin or the pregnant group but increased pPVN AVP mRNA expression in both virgin and pregnant rats. Thus we can conclude that IIH is not stimulating ACTH secretion via the CRH parvocellular PVN neurones, unless CRH has a permissive role, but more likely stimulates ACTH

secretion through the AVP neurones in the pPVN. Alternatively, IIH stimulates pPVN CRH/AVP neurones but selectively activates only AVP gene expression. Insulin signals the metabolic status to the hypothalamus but also causes hypoglycaemia. Several other neuropeptides signal metabolic status to the HPA axis; these include ghrelin, orexin and NPY. All of these signal lack of energy (unlike insulin). Insulin indicates abundant energy; in contrast, hypoglycaemia caused by insulin obviously reduces energy availability. Insulin has been shown to inhibit NPY/AgRP neurones (Porte *et al,* 2002), yet hypoglycaemia has been shown to activate orexin neurones which in turn activate NPY neurones (Jaszberenyi *et al*, 2001). These signals conflict, and it is not known whether it is insulin or hypoglycaemia that activates the HPA axis. This could be resolved by repeating the present experiments and infusing glucose i.v. to maintain normal glucose concentrations. The HPA axis has been shown to be unresponsive to orexin-A (Brunton *et al*, 2003) and NPY (Brunton *et al*, 2006) in late pregnancy; this includes also reduced stimulation of the pPVN CRH and AVP parvocellular neurones by NPY in late pregnancy (Brunton *et al*, 2006). The neuropeptide ghrelin is produced in the stomach and the ARC (Kojima *et al*, 1999). Ghrelin has also previously been shown to stimulate food intake and the HPA axis (Wren *et al*, 2002). The responsiveness of the HPA axis to ghrelin in late pregnancy is described in chapter 4. Chapter 4 also addresses the question of whether insulin's actions on the HPA axis are exerted in pregnancy through central ghrelin.

GHRELIN ACTIVATES THE HPA AXIS

4.1 Ghrelin

4.1.1 Ghrelin

Ghrelin is a 28-amino acid peptide hormone (Fig 4.1) that was discovered in 1999 as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R); it is predominantly produced in the stomach and potently stimulates food intake and growth hormone (GH) secretion (Kojima *et al*, 1999). It is produced and secreted by the A like cells in the oxyntic glands of the stomach (Sakata *et al*, 2002). The testis (Tanaka et al, 2001), placenta (Guabillo *et al*, 2001), kidney (Mori *et al*, 2000), pituitary (Korbonits *et al*, 2001), small intestine (Date *et al*, 2000), pancreas (Volante *et al*, 2002), lymphocytes (Hattori *et al*, 2001) and brain, specifically the arcuate nucleus (ARC) (Lu *et al*, 2001), also express low levels of ghrelin.

4.1.2 The growth hormone secretagogue receptor (GHS-R)

The GHS-R is a heterotrimeric GTP-binding protein (G-protein) first identified in 1996. There are two types of GHS-R: the functional type 1a and the non-functional type 1b. Both types are encoded by the same gene approximately 4.3kb in length but the mRNA undergoes alternative splicing (Howard *et al*, 1996). Types 1a and 1b consist of seven or five trans-membrane domains, respectively. Receptor 1a contains three intracellular and extracellular loops whereas receptor 1b contains two (Fig 4.2). Receptor type 1a is expressed mainly in the pituitary and at low levels in the thyroid gland, pancreas, spleen, myocardium and adrenal gland (Gnanapavan *et al*, 2002). Receptor 1b is widespread in many tissues including the stomach, oesophagus, intestine, liver, lung, muscle and testis (Gnanapavan *et al*, 2002). In the brain GHS-R's a and b are expressed by some specific hypothalamic neurones in the ARC, paraventricular nucleus (PVN), the ventromedial nucleus (VMH), dorsomedial

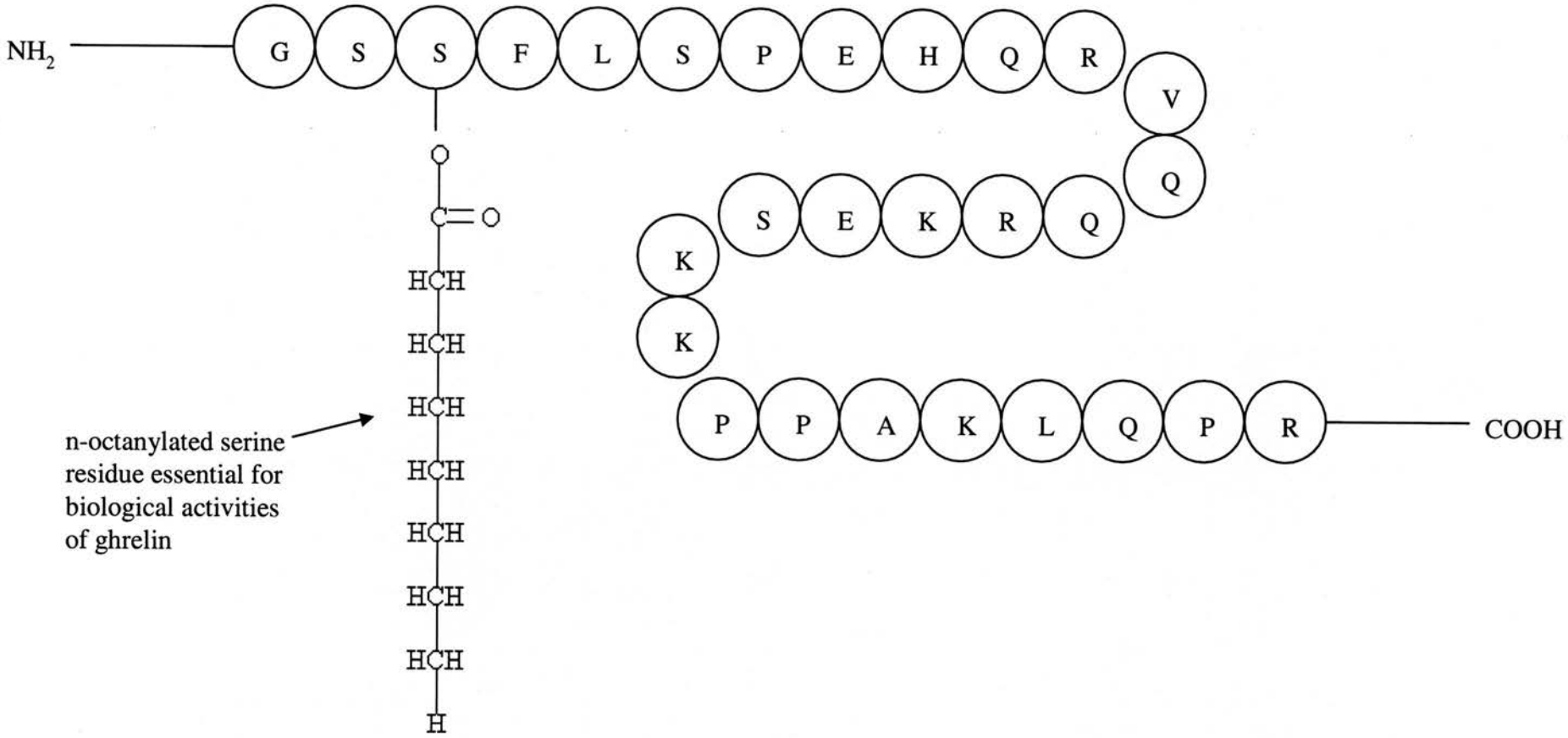


Figure 4.1: The structure of ghrelin (Kojima *et al*, 1999)

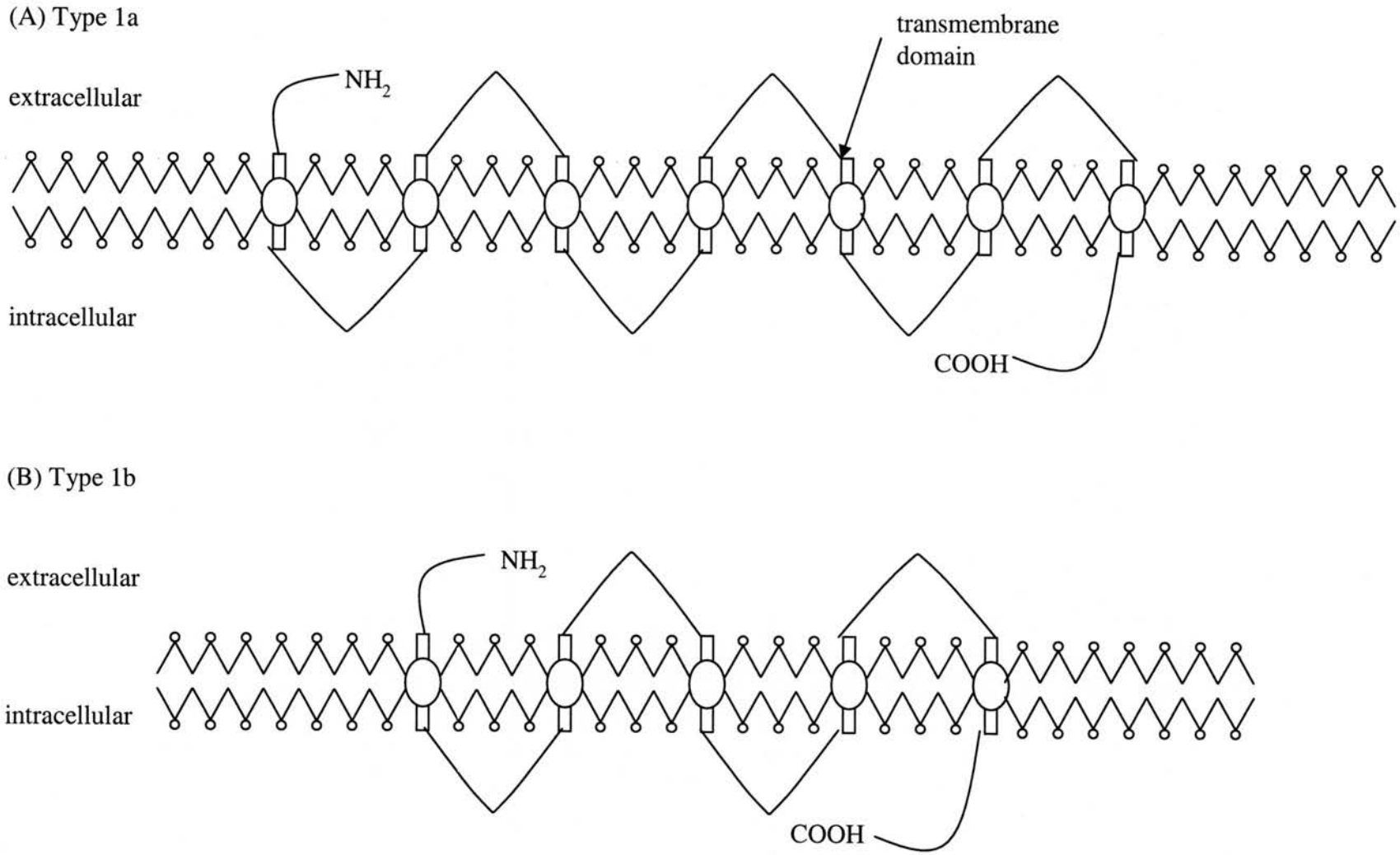


Figure 4.2: The structure of the growth hormone secretagogue receptor: type 1a and type 1b

hypothalamus (DMH) and lateral hypothalamic area (LHA) as well as by somatotrophs of the anterior pituitary gland (Guan *et al*, 1997).

The widespread distribution of the GHS-R explains the wide spectrum of ghrelin's actions which include stimulation of GH secretion, regulation of feeding and activation of the HPA-axis (Kojima *et al*, 2001).

4.1.3 The structure of ghrelin

There is no structural homology between ghrelin and the synthetic growth hormone secretagogues. Its amino acid sequence is highly conserved between species. The N-terminal portion of ghrelin is the active core of the molecule. Ghrelin bioactivity is ensured by post-translational acylation with octanoic acid at the 3rd serine residue (Fig. 4.1). Acylation permits ghrelin to cross the blood brain barrier (Guan *et al*, 1997) and is essential for its binding to its receptor (Banks *et al*, 2002). Transport of ghrelin across the blood brain barrier is a highly regulated and bi-directional process. It has been shown that ghrelin can be readily transported across the blood-brain barrier in the brain to blood direction but the quantity of its transport in the blood to brain direction is negligible (Banks *et al*, 2002). It must therefore be through a different mechanism that ghrelin exerts its central activities.

4.1.4. Ghrelin as a gut-brain peptide

It has recently been shown that ghrelin displays its central activity also via the vagus nerve as well as to a minor extent by transfer across the blood brain barrier (Date *et al*, 2002). Ghrelin receptors have been found in vagal afferent terminals in the stomach (Date *et al*, 2002). These fibres are the major anatomical linkage between the gastrointestinal (GI) tract and the nucleus of the solitary tract (NTS). Ghrelin induces NPY neurone activation in the nucleus of the solitary tract (NTS) and in the

ARC (Date *et al,* 2001). Thus it could influence appetite and energy expenditure via these actions.

Recently it has been shown that vagotomy (ablation of the vagus nerve) resulted in suppression of peripheral ghrelin-induced food intake and GH secretion (Date *et al*, 2002). Vagotomy did not interfere with GH or food intake when ghrelin was administered by i.c.v. injection (Date *et al*, 2002). This could mean there are two different independent ghrelin secreting systems: the central and the peripheral one. Ghrelin secreted from peripheral tissues could affect food intake through vagal afferents whereas ghrelin produced in the CNS acts through a direct effect on neurones.

4.1.5. Ghrelin expression in the brain

Ghrelin mRNA is expressed in the rat brain, mainly produced in the ARC and the NTS. Within the ARC ghrelin increases the expression of mRNA for neuropeptide Y (NPY), agouti related peptide (AgRP), somatostatin (SS) and growth hormone releasing hormone (GHRH) (Dickson *et al*, 1997). Ghrelin is also synthesised centrally in a group of neurones in the ARC (Cowley *et al*, 2003). In the brain ghrelin is mainly expressed in axons (Cowley *et al*, 2003) and is associated with dense core vesicles in pre-synaptic terminals. The axon terminals innervate several hypothalamic nuclei including the ARC, DMH, LHA and PVN (Cowley *et al*, 2003) and in all of these regions are neurones that express the GHS-R.

Ghrelin depolarises ARC NPY neurones and hyperpolarises POMC neurones (Cowley *et al,* 2003). This effect is most likely mediated by the GABAergic NPY/AgRP neurones (Cowley *et al,* 2003). These results can explain some of the effects of ghrelin on energy homeostasis. The activation of NPY/AgRP neurones

enhances the release of neuropeptides from these neurones to increase food intake and decrease energy expenditure (Fig.4.3).

4.1.6. Ghrelin's role in energy homeostasis

Ghrelin influences feeding via the orexin-NPY pathway (Dickson *et al*, 1997). NPY is one of the most potent central appetite stimulants (Stanley *et al*, 1986). Six receptor subtypes for NPY have been identified (Y1-Y6) and among these Y1 and Y5 are involved in the control of food intake (Fekete *et al*, 2002). AgRP, co-produced in the ARC NPY neurones, exerts a strong long-lasting stimulatory effect on feeding by antagonising the hypothalamic melanocortin anorexigenic system (Hahn *et al*, 1998). Ghrelin could also influence feeding by inhibition of leptin activity (Shintani *et al*, 2001). The regulatory role of leptin in the control of body weight is mediated at least partially through inhibition of the hypothalamic NPY/Y1 receptor pathway (Shintain *et al*, 2001). Ghrelin can reverse the leptin-induced down regulation of NPY mRNA expression in ARC neurones and thus inhibits leptin action (Nakazato *et al*, 2001).

4.1.7. Ghrelin and GH release

GH secretion from the anterior pituitary is regulated by the interaction between GHRH (which is stimulatory) and SS (which is inhibitory) (Roelfsema *et al*, 2001). A third factor responsible for regulating GH release is ghrelin, as simulated by artificial growth hormone secretagogues (Roelfsema *et al*, 2001). These stimulate GH secretion via the GHS-R independently of the GHRH and SS receptors. I.c.v. (Date *et al*, 2000) and i.v. (Seoane *et al*, 2000) administration of ghrelin increase GH secretion with no influence on other pituitary hormones (Kojima *et al*, 1999). In humans ghrelin strongly induces GH secretion more potently than GHRH. There are

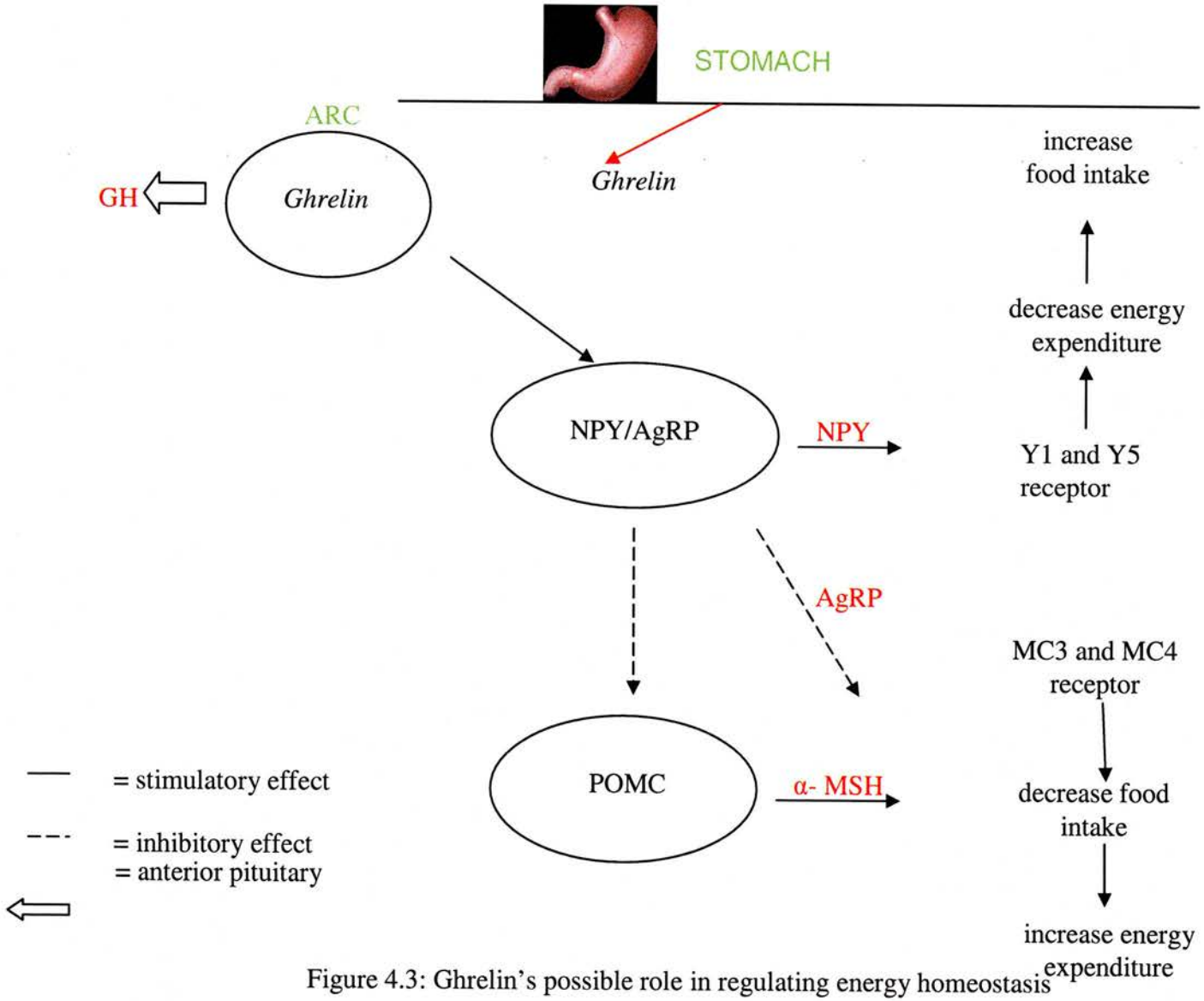


Figure 4.3: Ghrelin's possible role in regulating energy homeostasis

some reports that suggest also that ghrelin both in rats and humans could influence the secretion of some other pituitary hormones such as adrenocorticotropin (ACTH) and prolactin (Wren *et al*, 2001).

4.1.8. The effect of ghrelin on the HPA axis

CRH and AVP act together to stimulate ACTH release *in vivo* (Gillies *et al*, 1979). It has been shown that ghrelin can activate the HPA axis in rats when administered centrally, by i.c.v. injection, but not systemically, and it stimulates release of both CRH and AVP from hypothalamic explants (Wren *et al*, 2002). Administration of growth hormone secretagogues systemically resulted in a variable or absent ACTH response in rats (Thomas *et al*, 1997). In contrast both ghrelin and synthetic growth hormone secretagogues have been shown to stimulate ACTH and prolactin secretion following i.v. administration in humans (Arvat *et al*, 2001). The species disparity may reflect a different hypothalamic action. It has been shown that ghrelin releases NPY from rat hypothalamic explants *in vitro* (Wren *et al*, 2002); NPY neurones are a likely target for the action of ghrelin as they express the GHS-R (Guan *et al*, 1997). NPY neurones have been implicated in the orexigenic effect of ghrelin (Willesen *et al*, 1999) and in activating the HPA axis (Small *et al*, 1997). HPA axis activation by ghrelin would also lead to an increase in energy availability.

4.1.9 Aims of these experiments

Ghrelin appears to interact closely with the HPA axis and its regulatory pathways due to its effects at the level of CRH and AVP neurones in the pPVN (Wren *et al*, 2002). As discussed above ghrelin can activate the HPA axis in rats when administered centrally but not systemically (Wren *et al*, 2002). In late pregnancy the HPA axis is hyporesponsive to various physical and emotional stressors (Douglas *et*

al, 1998) and to i.c.v orexin A (Brunton *et al*, 2002); this is thought to be a consequence of reduced responsiveness of PVN CRH/AVP neurones (Brunton *et al*, 2002). Since ghrelin has been shown to activate the HPA axis when administered by i.c.v. injection we investigated if ghrelin activates the HPA axis in virgin and pregnant female rats when administered centrally. Central ghrelin has been shown to stimulate GH secretion; we measured blood glucose concentration as an indirect indicator of activation of mechanisms mobilising glucose including GH secretion. Ghrelin has also been shown to stimulate food intake. I.c.v. administration of orexin-A has revealed reduced responsiveness of the HPA axis in late pregnant rats but no differences in the stimulatory effects of orexin-A on behaviours, which indicates that loss of HPA axis stimulation is not a result of a general loss of central responses to orexin-A (Brunton *et al*, 2002).

Ghrelin has been shown to affect the release of many hormones including insulin; ghrelin decreases insulin secretion (Broglio *et al,* 2001). In Chapter 3 I showed that insulin activates the HPA axis similarly in virgin and pregnant rats. The main aims of the present experiment were: firstly to discover if centrally administered ghrelin would activate the HPA axis in female virgin rats and secondly to see if this response was altered in pregnant rats. In the experiment rats were blood sampled and brains processed for immunohistochemistry. Fos expression was evaluated in several areas including the pPVN to investigate activity in the CRH/AVP neurones and in the ARC, LHA, VMH, DMH to investigate circuitry regulating feeding responses to centrally administered ghrelin.

4.2 Methods

4.2.1 Animals

Female Sprague Dawley rats were used and housed individually after surgery. Rats were maintained as described in section 2.1.

4.2.2 Surgery

Rats were implanted with an intracerebroventricular and a jugular vein cannula. Surgery was performed under conditions described in section 2.3.

4.2.3 Experiment 1 – The effect of i.c.v. ghrelin on the HPA-axis

On the day of the experiment (day 21 of pregnancy) the cannulae were connected between 07:30-08:30h. The jugular vein cannula was attached to PVC extension tubing led out of the cage and connected to a 1ml syringe filled with heparinised saline (0.9% saline, 50 units/ml). Rats were left undisturbed for 90 minutes and a 0.55ml basal blood sample was taken. Thirty minutes after the basal blood sample rats were given either i.c.v. ghrelin (2nmols in 3µl) or vehicle (3µl artificial cerebro-spinal fluid, aCSF; pH 7.2, composition in mM: NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; NaH_2PO_4, 0.49; $CaCl_2$, 1.26; $MgCl_2$, 1.18), with gentle restraint over a period of about 30 seconds. Further blood samples were taken for glucose measurement (<100µl) at 10, 20, 30, 40, 50 and 60 minutes after i.c.v. infusion.

Blood sampling

Glucose measurements were taken throughout the experiment using a Roche Accuchek Active Meter. Blood samples at –30, 10, 30 and 60 minutes were placed into eppendorfs containing 50µl of chilled 5% EDTA. After each blood sample, blood was replaced with 0.9% sterile saline. Plasma was separated by centrifugation

and stored at -20°C until radioimmunoassay for ACTH and corticosterone. Ghrelin was given i.c.v. as it has previously been shown to activate the HPA axis when administered centrally but not peripherally.

Behavioural observations

The following behaviours were noted continually: inactive, grooming, oral motor activity, eating and drinking. Behavioural data were collected for 90 minutes; an event was classified as occurring if an animal spent $\geq$ 5 seconds exhibiting the behaviour.

Terminal procedures

Rats were killed by conscious decapitation 90 minutes after ghrelin (this time point has previously been shown as the optimum time for showing increased Fos expression in the ARC after ghrelin) (Niimi *et al,* 2001). Rats were examined post-mortem to check fetuses and to check i.c.v. cannula placement. Brains were processed for Fos immunohistochemistry on fixed sections and numbers of neurons expressing Fos in each region of interest were counted.

4.2.4 Fos immunohistochemistry

Coronal brain sections (15μm) were cut on the cryostat onto gelatine coated slides. With the aid of marker slides in consultation with the brain atlas (Konig *et al*, 1963), sections with PVN (Bregma – 1.8mm), SON (Bregma – 1.8mm), ARC (Bregma -2.3 mm), LHA (Bregma -2.3mm), VMH (Bregma -2.3mm) and DMH (Bregma -2.3mm) were selected. Fos immunohistochemistry was performed using the method described in section 2.5.3 with post-fixed sections. Fos positive cells were counted in each region with at least 3 sections counted per rat, and the observer blind to treatment.

4.2.5 ACTH radioimmunoassay

Plasma ACTH concentration was determined using a commercially available kit as described in section 2.7.3. The sensitivity of the ACTH assay was 5pg/ml. The intra-assay variation was <11%. All samples were measured in the same assay.

4.2.6. Corticosterone radioimmunoassay

Plasma corticosterone concentration was determined using a commercially available kit as described in section 2.8. Plasma corticosterone was measured directly in unextracted plasma (diluted 1:8 in assay buffer) using a double antibody radioimmunoassay. The sensitivity of the corticosterone assay was 0.8ng/ml. The intrassay variation was <6%. All samples were measured in the same assay.

4.2.7 Statistics

A two-way analysis of variance (ANOVA) was used to analyse behavioural and Fos data. A two-way ANOVA with repeated measures was used for blood sampling experiments. Each ANOVA that showed $p<0.05$ was followed by Student Newman Keuls multiple comparison tests, to identify differences between groups. A p value of less than 0.05 was considered statistically significant.

4.3 Results

4.3.1. The effects of i.c.v. ghrelin in pregnancy

Behavioural data

Analysis of behavioural data using a two-way ANOVA showed a statistically significant difference in eating behaviour among virgin and pregnant groups ($p<0.001$). Ghrelin significantly increased eating behaviour in both the virgin and late pregnant rats ($p<0.001$; two-way ANOVA) with a tendency for a greater effect in the late pregnant rats ($p=0.08$) (Fig 4.4). There was a significant increase in drinking in virgin rats ($p=0.019$; two-way ANOVA). There were no significant differences for grooming between groups (Fig 4.4).

Blood glucose concentration

Analysis of blood glucose concentration data using a two-way RM ANOVA showed a statistically significant difference in plasma glucose concentration among virgin and pregnant groups ($p<0.001$). Basal blood glucose concentrations were significantly lower in pregnant rats than in virgins ($p<0.001$; two-way RM ANOVA). I.c.v. injection of ghrelin increased plasma glucose in the virgin group ($p<0.001$; two-way RM ANOVA) within 10 min (7.4 ± 0.23 vs basal 5.88 ± 0.12 mmol/l) with glucose concentrations returning to basal by 20 min (Fig 4.5). Ghrelin significantly increased glucose concentration in pregnant rats ($p=0.002$; two-way RM ANOVA), within 10 min (5.02 ± 0.37 vs basal 4.02 ± 0.28 mmol/l) with glucose concentrations remaining elevated for 20 min (Fig 4.5). The overall effect of i.c.v. injection of ghrelin can be seen by the percentage change in glucose concentration from basal (Fig 4.6). Overall, the increase in blood glucose concentration was significantly greater in the pregnant/ghrelin group than all other groups at 30, 40, 50 and 60 min (Fig 4.6).

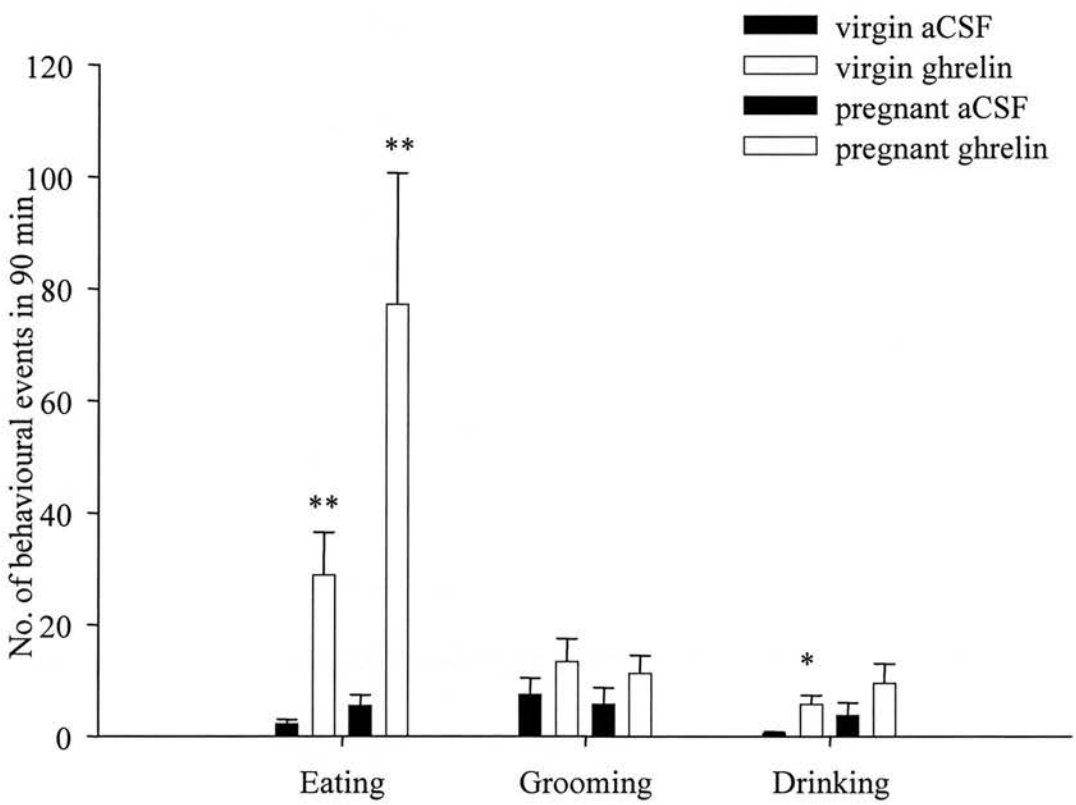


Figure 4.4: The effect of i.c.v. ghrelin on behaviours in virgin and pregnant rats. Values are the means ± SEM. Virgin/aCSF, n=8; virgin/ghrelin, n=10; pregnant/ aCSF, n=7; pregnant/ghrelin, n=8. A two-way ANOVA followed by Student Newman Keuls multiple comparison tests: **$p<0.001$, *$p<0.05$ vs aCSF.

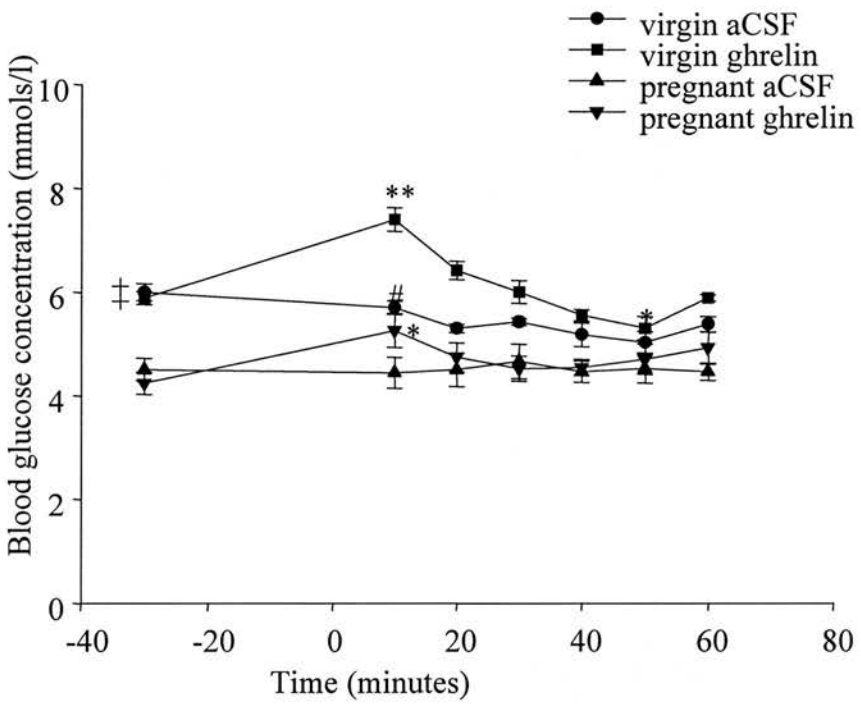


Figure 4.5: The effect of i.c.v. ghrelin on blood glucose concentration in virgin and pregnant rats. A basal blood sample was taken 30 min prior to i.c.v ghrelin (2nmols or aCSF). Further blood samples were withdrawn 10, 20, 30, 40, 50 and 60 min post-infusion. Values are group means ± SEM. Virgin/aCSF, n=4; virgin/ghrelin, N=; pregnant/aCSF, n=5; pregnant/ghrelin, n=6. Two-way ANOVA for repeated Measures followed by Student Newman multiple comparison tests: **$p<0.001$, *$p<0.05$ significantly different from basal; #$p<0.05$ significantly different from Virgin/ghrelin group at the same time point; +$p<0.05$ significantly different from pregnant.

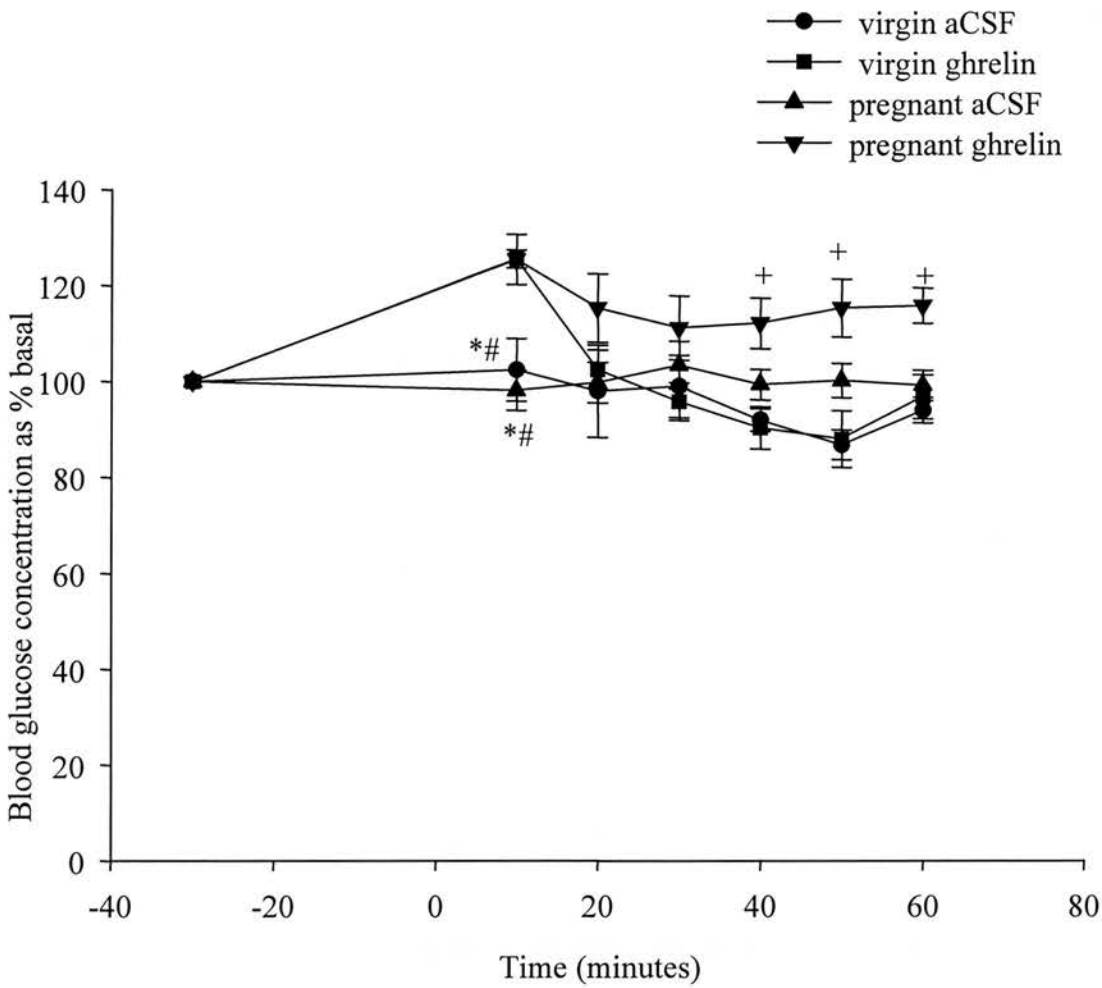


Figure 4.6: The effect of i.c.v. ghrelin on blood glucose concentration in virgin and pregnant rats. A basal blood sample was taken 30 min prior to i.c.v. ghrelin (2nmols or aCSF). Further blood samples were withdrawn 10, 20, 30, 40, 50 and 60 min post-infusion. Values are group means of plasma glucose concentrations expressed as % of basal ± SEM. Virgin/aCSF, n=4; virgin/ghrelin, n=6; pregnant/aCSF, n=5; pregnant/ ghrelin, n=6. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests: *$p<0.05$ significantly different from virgin/ghrelin; #$p<0.05$ significantly different from pregnant/ ghrelin at the same time point, +$p<0.05$ significantly different from all other groups at the same time point.

Plasma ACTH concentration

Analysis of plasma ACTH concentration data using a two-way RM ANOVA showed there was a statistically significant difference in plasma ACTH concentration among virgin and pregnant groups ($p<0.001$). Basal plasma concentrations of ACTH were not significantly different among groups. I.c.v injection of aCSF had no significant effect on plasma ACTH concentration except in the virgin group at 10 min ($p=0.004$; two-way RM ANOVA) which may have been due to stress of handling (Fig 4.7). I.c.v injection of ghrelin increased plasma ACTH concentration in the virgin group ($p=0.013$; two-way RM ANOVA) within 10 min (59.7 ± 5.3 vs basal 42.6 ± 6.8 pg/ml) (Fig 4.7) with concentrations remaining significantly elevated for 30 min (Fig 4.7). Ghrelin had no significant effect on plasma ACTH in the pregnant group (Fig 4.7). Thus, plasma ACTH concentration was significantly greater in the virgin group than in the pregnant rats at 30, 60 and 90 min after i.c.v. ghrelin.

Plasma corticosterone concentration

Analysis of plasma corticosterone concentration showed there was no overall statistically significant difference in plasma corticosterone concentrations among virgin and pregnant groups (Fig 4.8). I.c.v. injection of ghrelin or aCSF did not significantly increase plasma corticosterone concentration in any group (Fig 4.8). Data are expressed as the percentage change from basal as basal values were different among groups, although this was not statistically significant. Analysis of the data at the 10 minute sample point using a two-way ANOVA showed there was a statistically significant difference in plasma corticosterone concentrations among virgin and pregnant groups ($p=0.016$) (Fig.4.9). I.c.v. injection of ghrelin significantly increased plasma corticosterone only in the virgin group ($p=0.003$; two-way ANOVA) (181.3 ± 29.1 vs control 77.7 ± 0.86) (Fig.4.9).

Parvocellular paraventricular nucleus Fos expression

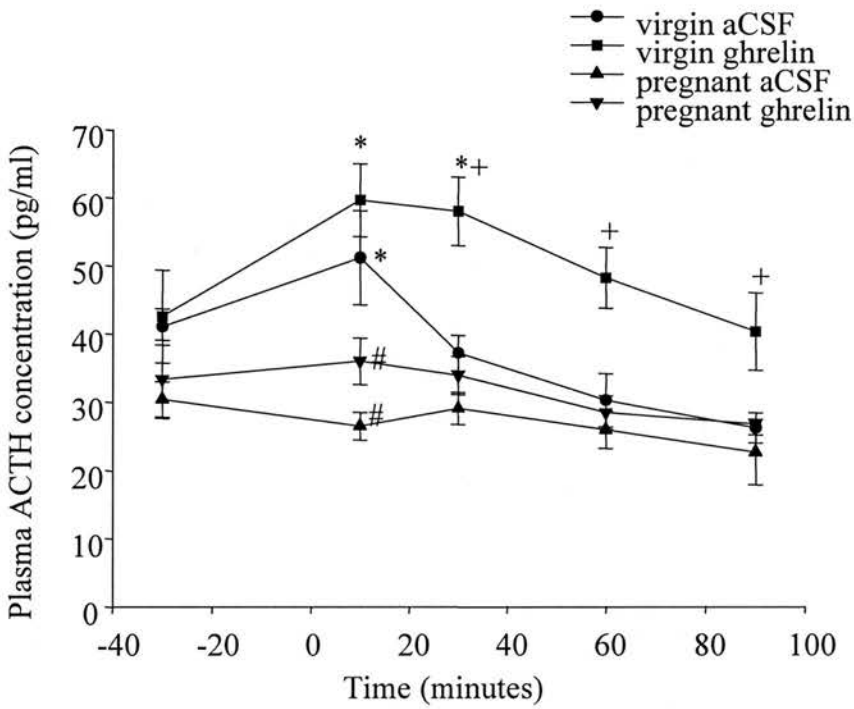


Figure 4.7: The effect of i.c.v. ghrelin on ACTH concentration in virgin and pregnant rats. A basal blood sample was collected 30 minutes before i.c.v. administration of ghrelin (2nmols or aCSF). Values are the group means ± SEM. Virgin/aCSF, n=4; virgin/ghrelin, n=6; pregnant/aCSF, n=5; pregnant/ghrelin, n=6. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests: *$p<0.05$ significantly different from basal, #$p<0.05$ significantly different from virgin/ghrelin group at the same time point, +$p<0.05$ significantly different from all other groups at the

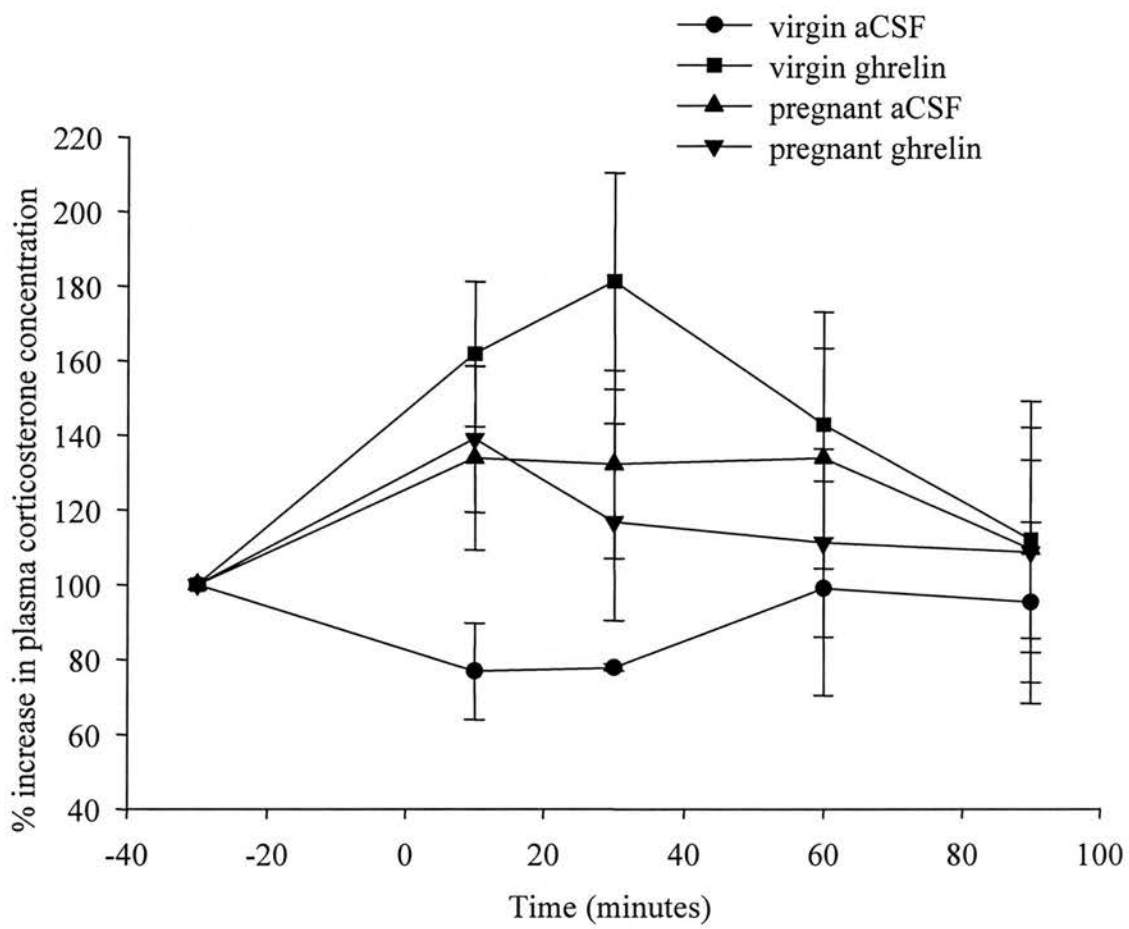


Figure 4.8: The effect of i.c.v. ghrelin on corticosterone concentration in virgin and pregnant rats. A basal blood sample was collected 30 minutes before i.c.v. administration of ghrelin (2nmols). Further blood samples were withdrawn 10, 30, 60 and 90 (trunk) minutes post-infusion. Values are the group means ± SEM. Virgin/aCSF, n=4; virgin/ Ghrelin, n=6; pregnant/aCSF, n=5; pregnant/ghrelin, n=6. Two-way ANOVA for repeated measures. Data are shown as the % of basal values.

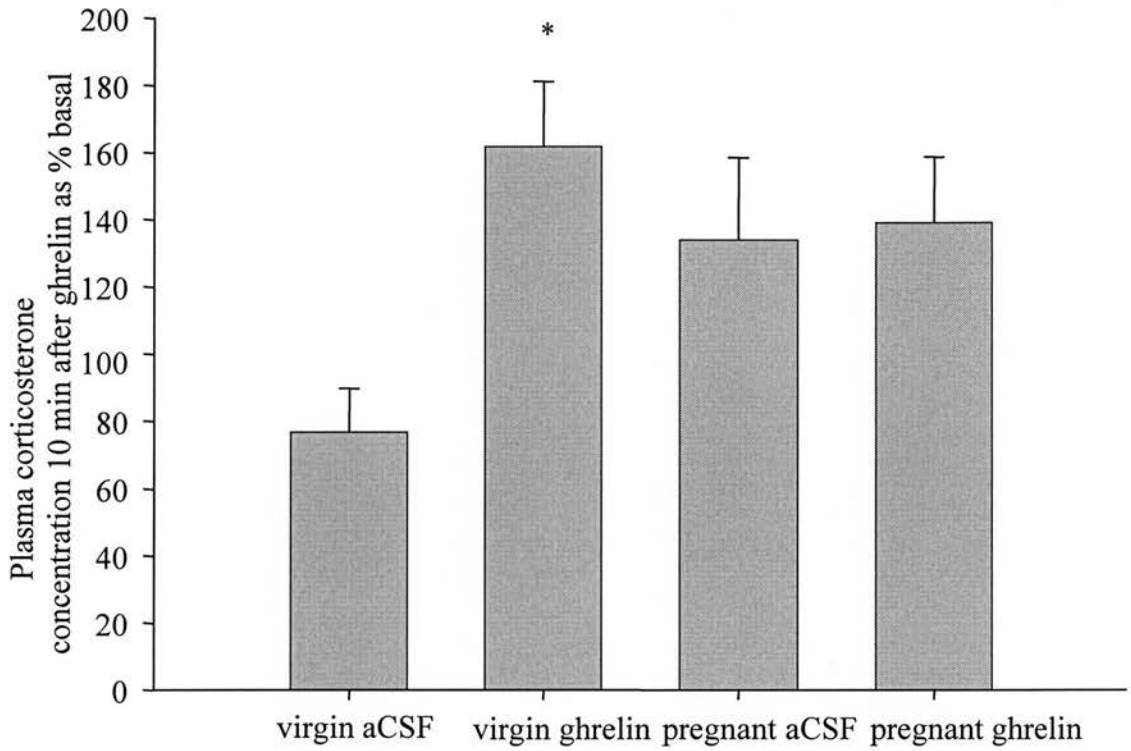


Figure 4.9: The effect of i.c.v. ghrelin on corticosterone concentration in virgin and pregnant rats 10 minutes after i.c.v. ghrelin. Values are the group means ± SEM. Virgin/aCSF, n=4; virgin/ghrelin, n=6; pregnant/aCSF, n=5; pregnant/ghrelin, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test. *$p<0.05$ vs virgin aCSF. Data are shown as the % of basal values.

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p = 0.01$) (Fig 4.10). The number of Fos positive cells per pPVN profile of virgin rats given ghrelin was significantly greater (11-fold) than in the pregnant ghrelin-treated group (Fig 4.10). I.c.v. injection of ghrelin significantly increased Fos expression in the virgin group ($p = 0.003$) (11.9± 2.6 vs control 3.1 ± 1.2 Fos positive cells per pPVN profile) (Fig 4.10 and Fig 4.11), but had no effect in the pregnant group.

Arcuate nucleus Fos expression

Analysis of numbers of Fos-positive cells in the ARC using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p = 0.012$). The number of Fos-positive cells in the ARC of virgin rats given i.c.v. ghrelin was significantly greater (5-fold) than in the pregnant ghrelin-treated group (Fig 4.12 and Fig 4.13). I.c.v. injection of ghrelin significantly increased Fos expression in the virgin group ($p<0.05$) (Fos positive cells/section: 16.1 ± 4.7 vs control 3.0 ± 1.6), but not in the pregnant group (Fig 4.12 and Fig 4.13).

Lateral hypothalamic area Fos expression

Analysis of Fos expression in the LHA using a two-way ANOVA showed there was a statistically significant difference in Fos expression among aCSF and ghrelin-treated groups ($p=0.021$) (Fig 4.14). Ghrelin increased Fos expression in both virgin and pregnant rats, however there was not a statistically significant difference in Fos expression between virgin and pregnant groups for either ghrelin or aCSF treatment (Fig 4.14).

Dorso-medial hypothalamus Fos expression

Analysis of Fos expression in the DMH using a two-way ANOVA showed there was

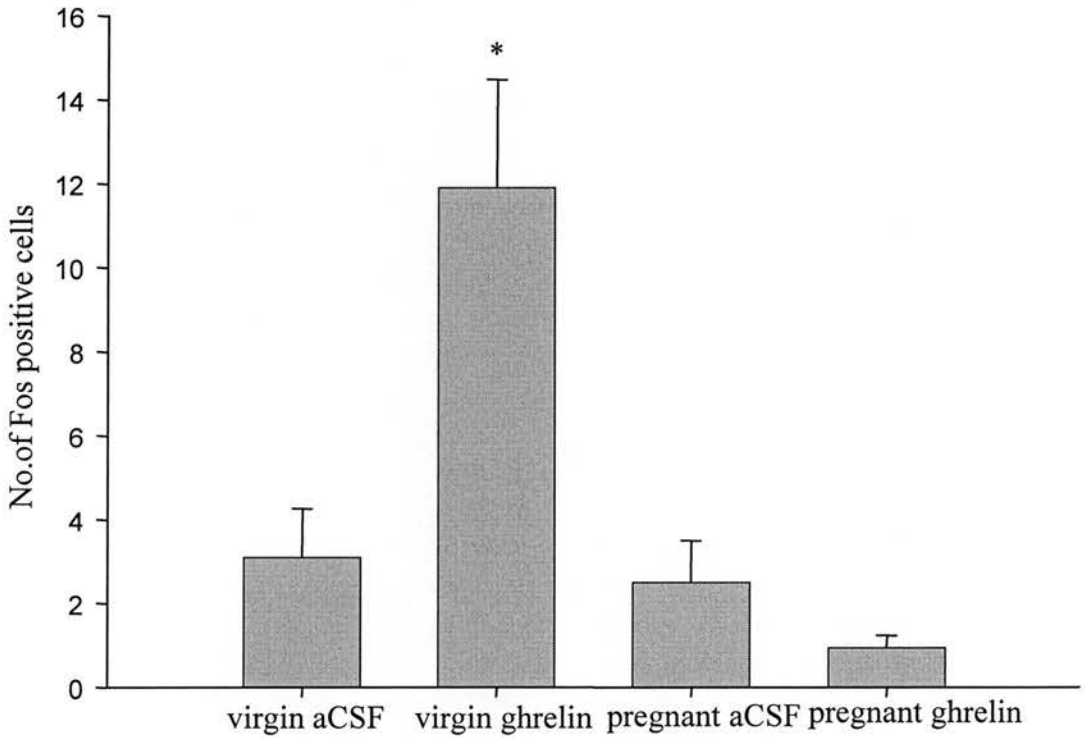


Figure 4.10: The effect of i.c.v. ghrelin on Fos counts in the pPVN in virgin and pregnant rats. Rats were killed by conscious decapitation 90 minutes post-injection of ghrelin or aCSF.Values are the group means ± SEM of the mean counts of positive cells per pPVN profile over three sections per rat. Virgin/aCSF, n=9; virgin/ghrelin, n=11; pregnan/aCSF, n=7; pregnant/ghrelin, n=8. A two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *p=0.003 vs all other groups.

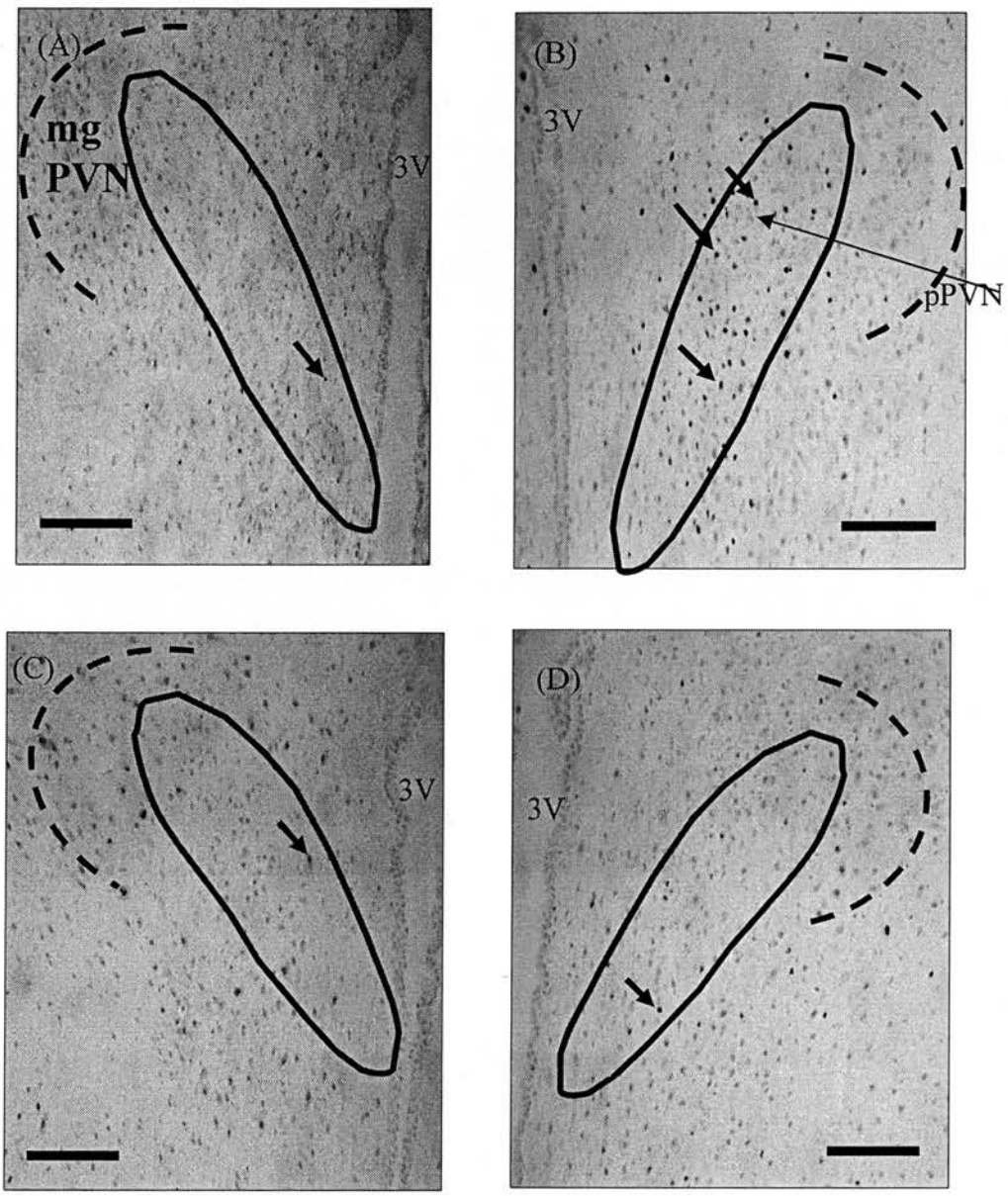


Figure 4.11: Representative photomicrographs of coronal sections through the PVN of cryostat sections (15μm) in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry for (A) virgin aCSF; (B) virgin ghrelin; (C) pregnant aCSF; (D) pregnant ghrelin. Small arrows indicate Fos-positive nuclei; the solid line outlines the parvocellular PVN (pPVN), and the dashed line outlines the magnocellular PVN (mgPVN).
Scale bar = 100μm. 3V = 3rd ventricle.

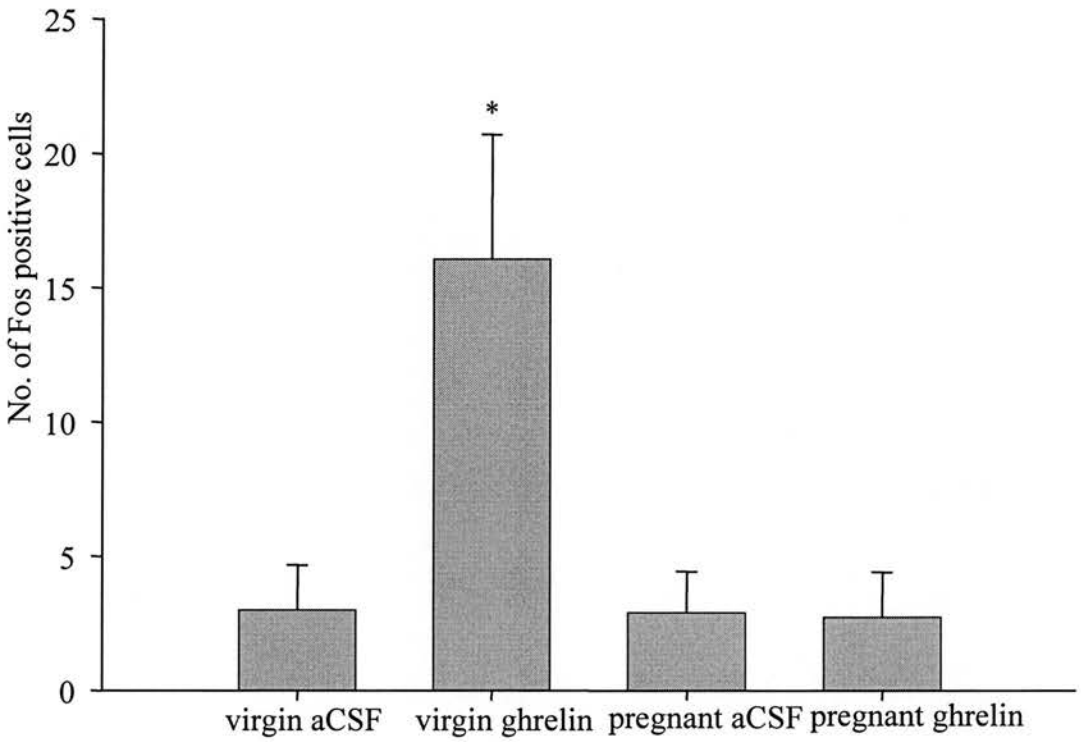


Figure 4.12: The effect of i.c.v. ghrelin on Fos positive cell counts in the ARC in virgin and pregnant rats. Rats were killed by conscious decapitation 90 minutes post-injection of ghrelin or aCSF. Values are the group means ± SEM of the mean counts of Fos positive cells over three sections. Virgin/aCSF, n=9; virgin/ghrelin, n=11; pregnant/aCSF, n=7; pregnant/ghrelin, n=8. A two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs all other groups.

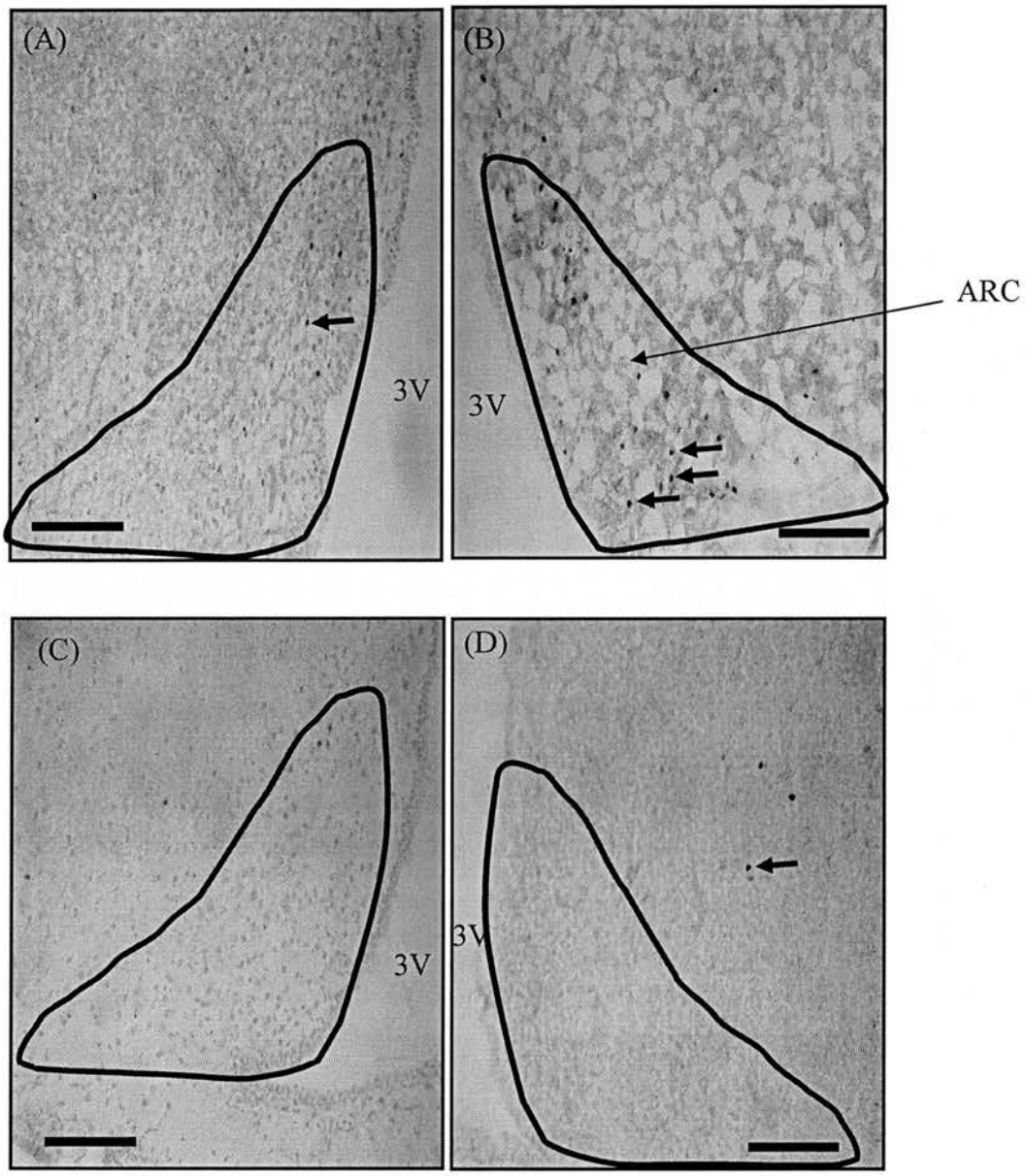


Figure 4.13: Representative photomicrographs of coronal sections through the ARC of cryostat sections (15μm) in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry for (A) virgin aCSF; (B) virgin ghrelin; (C) pregnant aCSF; (D) pregnant ghrelin. Small arrows indicate Fos-positive nuclei; the solid line outlines the arcuate nucleus. Scale bar = 100μm. 3V = 3rd ventricle.

a statistically significant difference in Fos expression among virgin and pregnant groups (p=0.001). I.c.v. injection of ghrelin significantly increased Fos expression in the virgin group ($p<0.05$) (Fos positive cells/ profile: 8.1 ± 1.4 vs control 2.2 ± 0.8) (Fig 4.15) and in the pregnant group ($p <0.05$) (2.5 ± 0.8 v control 0.3 ± 0.2) (Fig 4.15). The number of Fos positive cells in the DMH in the virgin/ghrelin group was significantly greater (4-fold) than in the pregnant/ghrelin group (Fig 4.15).

Ventro-medial hypothalamus Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among aCSF and ghrelin treated groups ($p = 0.046$) (Fig 4.16). However, post-hoc analysis showed there was not a statistically significant difference in Fos expression between the respective virgin and pregnant groups.

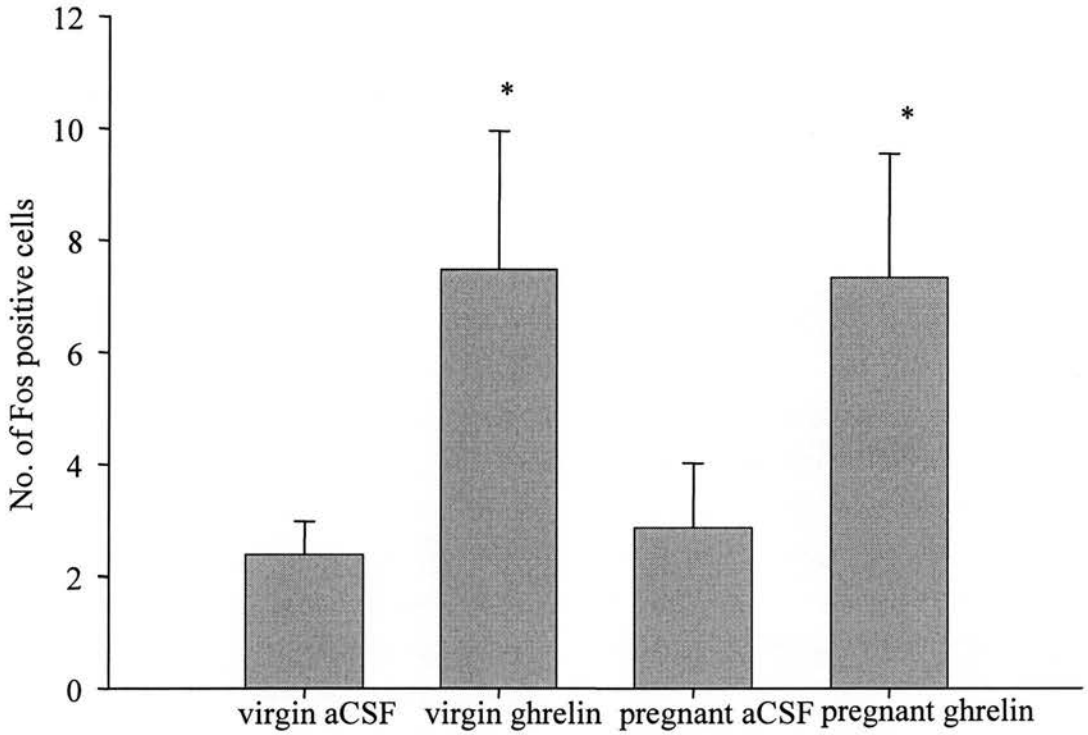


Figure 4.14: The effect of i.c.v. ghrelin on Fos expression in the LHA in virgin and pregnant rats. Rats were killed by conscious decapitation of ghrelin. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/Acsf, n=7; virgin/ghrelin, n=10; pregnant/aCSF, n=7; pregnant/ghrelin, n=8. Two-way ANOVA: *p=0.021, significant effect of ghrelin.

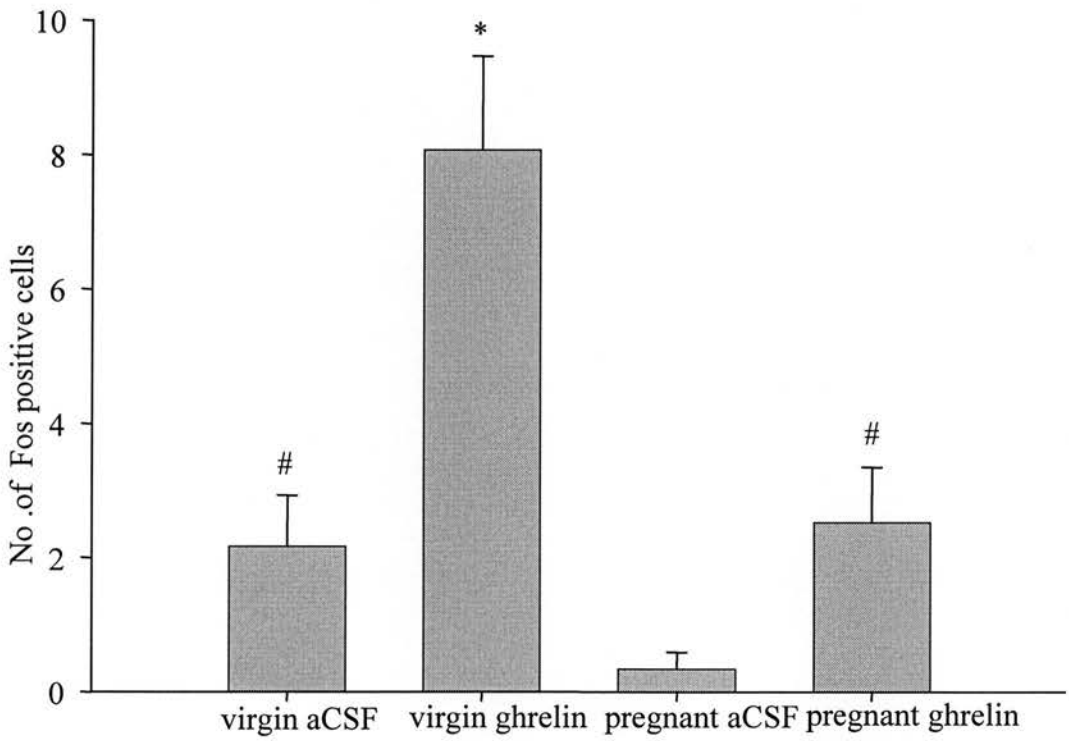


Figure 4.15: The effect of i.c.v. ghrelin on Fos counts in the DMH in virgin and pregnant rats. Rats were killed by conscious decapitation 90 minutes post-injection of ghrelin or aCSF. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=9; virgin/ghrrelin, n=11; Pregnant/aCSF, n=7; pregnant/ghrelin, n=8. A two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs all other groups. #$p<0.05$ vs pregnant aCSF.

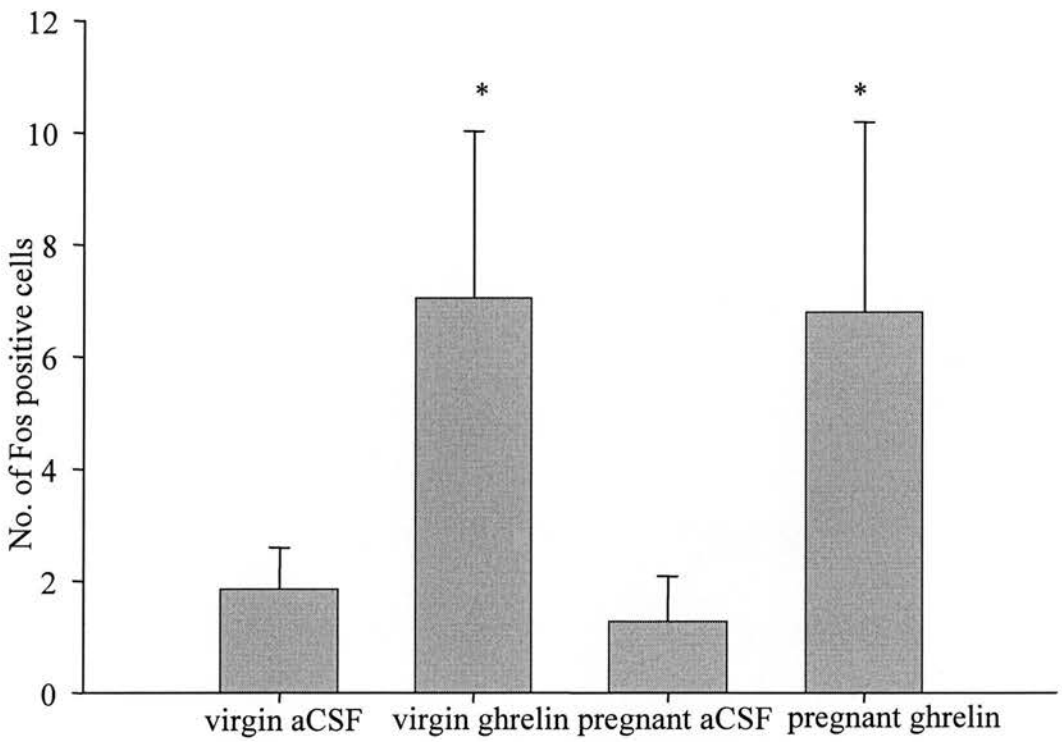


Figure 4.16: The effect of i.c.v. ghrelin on Fos counts in the VMH in virgin and pregnant rats. Rats were killed by conscious decapitation 90 minutes post injection of ghrelin or aCSF. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=9; virgin/ghrelin, n=11; Pregnant/aCSF, n=7; pregnant/ghrelin, n=8. Two-way ANOVA: *P<0.05 vs aCSF

4.4 Discussion

4.4.1. The effect of i.c.v. ghrelin on food intake

The results show that centrally administered ghrelin stimulates eating behaviour in both virgin and pregnant rats. This effect has been shown previously in male rats (Wren *et al*, 2001). Ghrelin also stimulated drinking behaviour in virgin and pregnant rats although not significantly in the pregnant group; this may relate to the tendency of the pregnant rats to spend more time eating after ghrelin, and to the greater drinking behaviour in the vehicle-treated pregnant rats. Other studies have shown no significant changes in drinking or grooming in response to ghrelin administration (Ishizaki *et al*, 2002). We found no significant effect of ghrelin on grooming.

4.4.2. The effect of i.c.v. ghrelin on blood glucose concentration

The results show that i.c.v. ghrelin significantly increases blood glucose concentration to a similar maximal extent in both virgin and pregnant rats, although the increase lasted for longer in the pregnant ghrelin-treated rats (Fig 4.6). Hyperglycaemia and a decrease in insulin concentration have previously been shown in ghrelin-treated rats (Broglio *et al*, 2001). Increase in GH secretion stimulated by ghrelin may explain the hyperglycaemia seen in both virgin and pregnant rats. GH suppresses the ability of insulin to stimulate uptake of glucose in peripheral tissues and enhances glucose synthesis in the liver (Hayes *et al*, 2001). Consequently, GH increases blood glucose (Ghanaatt *et al*, 2005), and has two other distinct metabolic effects; a direct effect of GH stimulates fat cells to breakdown triglyceride and suppresses their ability to take up circulating lipids (Harant *et al*, 1994), and an indirect effect mediated primarily by insulin like growth factor I (IGF-I), a hormone secreted by the liver and other tissues in response to GH. GH is one of a battery of

hormones, which includes glucocorticoids that serves to maintain blood glucose within a normal range.

Basal blood glucose concentration was lower in pregnant rats compared to virgins; this corresponds with previous studies (Rossi *et al*, 1993). This effect is probably due to increased metabolic demands of the fetuses on the mother, as well as the fact that blood volume is also increased in pregnancy.

The increased blood glucose levels in response to i.c.v. ghrelin returned to basal levels by 20-30 minutes. GH was not measured neither was insulin in this experiment however so we do not know the reason for the stimulatory effect of blood glucose. Corticosterone seemed not responsible for the increase in blood glucose as it was not increased by ghrelin in pregnant rats. However, the increase in blood glucose after i.c.v. ghrelin in pregnant rats showed that sensitivity to this action is retained in late pregnancy.

4.4.3 The effect of i.c.v. ghrelin on the HPA axis

The results show that ghrelin significantly increased plasma ACTH concentration in virgin female rats, as shown previously in male rats (Wren *et al*, 2002). Virgin rats given aCSF showed a significant transient increase in plasma ACTH concentration at 10 minutes which could be due to non-specific stress associated with the i.c.v. injection. Virgin rats given i.c.v. ghrelin showed a significant sustained increase in plasma ACTH concentration, for at least 90 minutes, which was more prolonged than in rats given aCSF. No effect of i.c.v. ghrelin or aCSF on ACTH secretion was seen in late pregnant rats. Virgin rats given i.c.v. ghrelin had significantly greater plasma ACTH concentrations at 30, 60 and 90 minutes following ghrelin injection. Thus, ACTH responses to i.c.v. ghrelin are suppressed in late pregnancy, which corresponds with previous studies of orexin-A actions in pregnancy (Brunton *et al*,

2003). An overall significant increase in plasma corticosterone concentration was not demonstrated following ghrelin administration in either virgin or pregnant rats, but there was a clear trend for plasma corticosterone concentration to increase in virgin rats given ghrelin compared to virgin controls, which corresponds with previous studies in male rats (Wren *et al,* 2002). In contrast there was no tendency for plasma corticosterone concentration to increase in late pregnant rats after i.c.v. ghrelin.

One reason for reduced ACTH responsiveness in late pregnancy has been shown to be reduced responsiveness of the pPVN CRH/AVP neurones in pregnancy. Here, i.c.v. ghrelin significantly increased the number of Fos positive cells in virgin rats in the pPVN compared with aCSF-treated rats. There was also a significantly greater number of Fos-positive cells in the pPVN in virgin rats compared to late pregnant rats, which did not respond compared to aCSF-treated pregnant rats. This suggests there is reduced responsiveness of the CRH/AVP neurones in pregnancy in the parvocellular PVN, as these neurones comprise a major population of the pPVN neurones (e.g. 91%; Arima *et al,* 2000).

It is also known that ghrelin activates NPY neurones in the ARC that project to the pPVN (Dickson *et al*, 1997) and NPY has been shown to activate the HPA axis (Small *et al,* 1997, see chapter 6). Here, ghrelin significantly increased the number of Fos-positive cells in virgin rats in the ARC. There was also a significantly greater number of Fos positive cells in the ARC in virgin rats given i.c.v. ghrelin compared to pregnant rats, which did not respond to i.c.v. ghrelin. As ARC NPY neurones project to the pPVN to activate the HPA axis, the lack of a response of ARC NPY neurones to i.c.v. ghrelin could be a reason why there is suppressed responsiveness of the HPA axis to ghrelin in pregnancy. This is investigated further in chapter 6.

Ghrelin has also been shown to induce Fos expression in GHRH (Hewson *et al*, 2000), and somatostatin neurones (Lawrence *et al*, 2002). Fos expression in POMC

neurones in the ARC has been shown to be unaffected by ghrelin (Kingzig *et al,* 2006). We do not know the phenotype of the neurones that were activated in virgin rats in the ARC in response to i.c.v. ghrelin in the present experiment. Further studies using double immunohistochemistry are needed to identify the neuropeptide phenotype of neurones activated by i.c.v. ghrelin. Previous studies using double labelling have shown that ARC NPY neurones are activated in response to i.c.v. ghrelin (Huda *et al*, 2006), so it is likely that the ARC neurones activated in virgin rats in the present study produce NPY/AgRP.

4.4.4. Activation of feeding circuitry by i.c.v. ghrelin

The LHA is a key feeding centre (Cripps *et al,* 2005), and the finding in the present study that i.c.v. ghrelin increased the number of Fos positive cells in the LHA in both virgin and late pregnant rats may account for the similar effects of i.c.v. ghrelin on eating behaviour in virgin and pregnant rats. Ghrelin has also been shown to stimulate food intake (Kojima *et al,* 1999), and this effect on food intake is mediated through NPY neurones (Dickson *et al,* 1997). NPY neurones in the NTS are also activated in response to i.c.v. ghrelin (Huda *et al*, 2006). NPY neurones project to orexin containing neurones in the LHA (Broberger *et al,* 1998). It seems possible that i.c.v. ghrelin induced Fos expression in the LHA via activation of ARC NPY neurones in virgin rats, but in pregnant rats ghrelin may have acted directly or via NTS NPY neurons (Lawrence et al, 2002). Activation of Fos expression in the LHA by i.c.v. ghrelin in both virgin and pregnant rats corresponds with the finding that eating behaviour was stimulated in both virgin and pregnant rats in response to i.c.v. ghrelin. It would be interesting to evaluate actions of i.c.v. ghrelin on NTS neurones in virgin and late pregnant rats.

It has been shown that ghrelin induces Fos expression in the DMH (Lawrence *et al,* 2002). Dense projections of ARC NPY/AgRP neurones innervate the DMH (Graham *et al,* 2003). I.c.v. ghrelin significantly increased the number of Fos positive cells in the DMH in both virgin and pregnant rats compared to the control groups. The basal expression of Fos expression in the DMH was lower in pregnant animals compared to virgin animals and pregnant rats given i.c.v. ghrelin showed significantly less Fos expression than virgin rats given i.c.v. ghrelin. The DMH contains a high proportion of NPY neurones (about 70%) (Chen *et al*, 2004). Both the DMH and VMH are essential for basal and induced ACTH secretion (Fizol *et al*, 1977). Consequently, the reduced Fos responses in the DMH after i.c.v. ghrelin in late pregnant rats might contribute to the suppressed HPA axis response. Conversely, the intact VMH Fos responses indicate that VMH activation is not sufficient to drive the HPA axis in late pregnancy. Perhaps the reduced increase in Fos expression in the DMH after i.c.v. ghrelin in late pregnant rats reflects failure of ARC NPY neurones to respond to i.c.v. ghrelin.

It has also been shown that ghrelin induces Fos expression in the VMH after administration of a growth hormone secretagogue (Lawrence *et al,* 2002) or ghrelin, although the effect of ghrelin was not significant (Lawrence *et al,* 2002). It is known that in the centre of the VMH there are glucose responsive neurones (Ono *et al,* 1982). The VMH responds to an increase in blood glucose levels (Borg *et al,* 2003) and has also previously been identified as a "satiety centre" (King *et al,* 2006). We have shown that i.c.v. ghrelin increased the number of Fos positive cells in the VMH in both virgin and pregnant rats compared to control groups. This may have been due entirely to a central action of ghrelin, perhaps a result of the hyperglycaemia following ghrelin.

ARC NPY neurones are also importantly implicated in the central regulation of appetite (Small *et al,* 1997). The action of ghrelin in virgin rats in stimulating both ARC nucleus neurones, as detected by Fos expression, and eating behaviour is consistent with this. However, in late pregnant rats ghrelin strongly stimulated eating behaviour without activating ARC neurones. These findings indicate that ghrelin can stimulate eating in other ways than by stimulation of the ARC NPY neurones. The mechanism could be by a direct action of ghrelin on the LHA (which contains orexin neurones) and the DMH (which comprise 70% of NPY neurones). Indeed, regarding the VMH, ghrelin also increased Fos in both the virgin and pregnant rats. Here ghrelin was shown to transiently increase blood glucose, the VMH neurones are glucose sensing, their activation after ghrelin may reflect the fluctutations in blood glucose.

4.4.5 Summary

In summary, the experiments have shown that i.c.v. ghrelin activates the HPA axis in virgin but not in late pregnant rats, yet it increased eating behaviour and blood glucose concentration in both virgin and pregnant rats. Fos expression was stimulated by i.c.v. ghrelin in both virgin and pregnant rats in the LHA, VMH and DMH which has already been shown in male rats (Lawrence *et al,* 2002). These findings also correspond with the location of the GHS-R (Cowley *et al,* 2003). In pregnancy HPA axis responses to i.c.v. ghrelin were suppressed. Fos expression was not stimulated by central ghrelin in the pPVN or the ARC in pregnant rats. Reduced expression in the pPVN neurones indicates that the reduced ACTH response was due to lack of activation of the pPVN CRH and AVP neurones. Therefore the responsiveness of the HPA axis to i.c.v. ghrelin is reduced in pregnancy but blood glucose and feeding responses are still intact. Thus the loss of stimulatory actions of

ghrelin in pregnancy on the HPA axis is not a result of a generalised loss of central responses to i.c.v. ghrelin. The finding that there were reduced responses of the HP axis to i.c.v. ghrelin which were not seen in response to i.v. insulin (Chapter 3) suggests that insulin's actions on the HPA axis are not exerted via central ghrelin. There have been several ideas as to the mechanisms of HPA axis hyporesponsiveness to stressors in late pregnancy. It has previously been shown that enhanced negative feedback inhibition by glucocorticoids does not underlie the reduced responsiveness of the HPA-axis to stress at the end of pregnancy (Johnstone *et al,* 2000) and reduced forward drive is more likely to account for the reduced activity (Johnstone *et al,* 2000). This may involve inhibition by a central opioid mechanism that emerges in pregnancy (Douglas *et al,* 1998). Possible sources of endogenous opioid that could regulate pPVN CRH/AVP neurones include noradrenergic NTS neurones which coexpress pro-enkephalin-A (pENK-A) mRNA (Ceccatelli *et al,* 1989). pENK-A mRNA expression is increased in the NTS in late pregnancy and evidently inhibits CRH neurones following administration of interleukin-1β (Brunton *et al,* 2005). NPY-containing neurones project from the NTS to the PVN. It was not known whether this opioid mechanism would interfere with NPY signalling to the pPVN CRH/AVP or ARC neurones (see Chapters 5 and 6). We aimed to investigate further the mechanisms of reduced HPA axis responses in pregnancy, so in Chapter five we have examined central effects of orexin-A in pregnancy and the role of endogenous opioids to investigate this further, since orexin-A has been shown to activate the HPA axis in part via NPY neurones (Jasberenzyi *et al,* 2001). A possible role for endogenous opioids in the suppressed pPVN neurone and ACTH responses to ghrelin remains to be investigated. The results lead us to consider that the central actions of ghrelin on the HPA axis involve ARC neurone activation (since this was absent in pregnant rats which did not show an HPA axis response), while the actions of ghrelin

on eating are not mediated by stimulatory actions in the ARC but may be mediated by direct actions on the LHA and VMH, with a lesser role for the DMH. It should be remarked that Fos expression allows detection of neurones that have been excited (Hoffman *et al,* 1993) and different inhibitory actions of ghrelin on pools of neurones between pregnant and virgin rats would have gone undetected.

OREXIN

5.1.1 The Orexins

Orexins A and B are neuropeptides originally isolated from the rat hypothalamus by two different groups of investigators (De Lecea *et al*, 1998; Sakurai *et al*, 1998). Two G-protein coupled receptors for orexins A and B were discovered and are named the orexin receptor 1 (OX1-R) and the orexin receptor 2 (OX2-R) (De Lecea *et al,* 1998; Sakurai *et al*, 1998).

The orexins have been implicated in playing a role in the regulation of energy balance and feeding (Spinazzi *et al,* 2006) but they have also been importantly involved in the regulation of the sleep-wake cycle (Lin *et al,* 1999). Orexin receptor knock out mice with a non functional OX2-R, display a narcolepsy like condition (Chemelli *et al,* 1999; Lin *et al,* 1999). It is thought that orexin may regulate sleep by interacting through aminergic and cholinergic systems (Lin *et al,* 1999), and inhibition of orexin neurones through GABAergic inputs from pre-optic and basal forebrain areas is thought to be crucial for sleep initiation (Eggerman *et al,* 2003). Narcoleptic humans have been shown to lack orexin-containing neurones in the hypothalamus and orexin in cerebrospinal fluid (Nishino *et al,* 2000). As well as the sleep-wake cycle and feeding the orexins can regulate blood pressure and heart rate and can also activate the HPA axis (Taheri *et al,* 2001). The fact that orexin can activate the HPA axis is very interesting as many peptides that are involved in the central regulation of feeding (e.g. leptin and neuropeptide Y (NPY) also help control activity of the HPA axis (Crawley *et al,* 1994).

5.1.2. Orexin Biosynthesis

The orexins are derived from the post-translational cleavage of an amino acid precursor prepro (pp)-orexin. After detachment of the N-terminal 33 amino acid residue pp-orexin is converted to pro-orexin, which is then cleaved to yield one molecule each of orexin-A and orexin-B.

Orexin-A

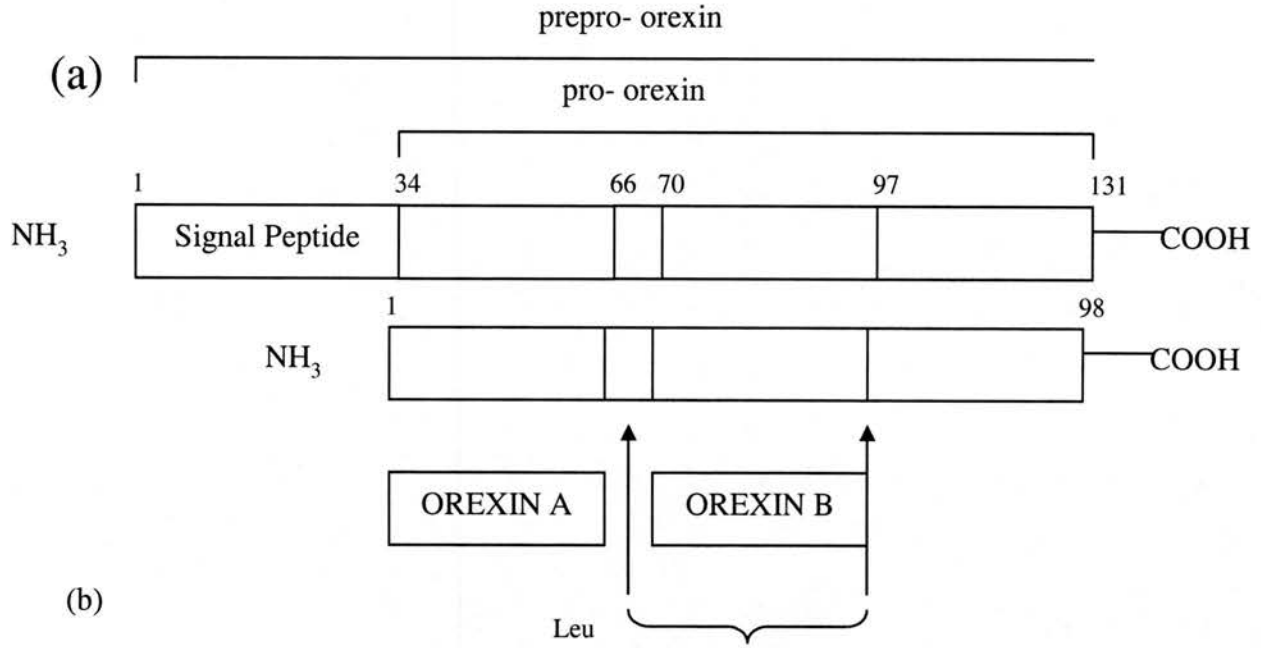
Orexin-A is a 3.5kDa 33 amino acid peptide. It possesses a N-terminal residue, a C-terminal and two disulphide bridges between adjacent cysteine residues (Fig 5.1).

Orexin-B

Orexin-B is a 2.9kDa 28 amino acid peptide. Orexin-A is more stable than orexin-B, Orexin-A is found in higher concentrations in tissue and blood than orexin-B (Kastin *et al*, 1999). Orexin-A can cross the blood brain barrier unlike orexin-B (Kastin *et al,* 1999).

5.1.3 Orexin receptors and their signalling mechanism

The orexin receptors are seven transmembrane domain G-protein coupled receptors (Sakurai *et al,* 1998). OX1-R binds only orexin-A, whereas the OX2-R binds both orexin-A and orexin-B. Activation of orexin receptors increases intracellular calcium concentration (Smart *et al,* 1999). Activation of phospholipase C catalyses the breakdown of phosphatidyl inositol to inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) and IP3 and increases calcium release from intracellular calcium stores and also activates PKC (Zhin *et al,* 2003). PKC and the increased intracellular calcium open voltage-gated calcium channels causing membrane depolarisation (Fig. 5.2). As well as activating the orexin receptor



After removal of the signal peptide pro-orexin is cleaved by a prohormone convertase at sites (shown by an arrow) with basic amino acid residues to produce orexin A and orexin B

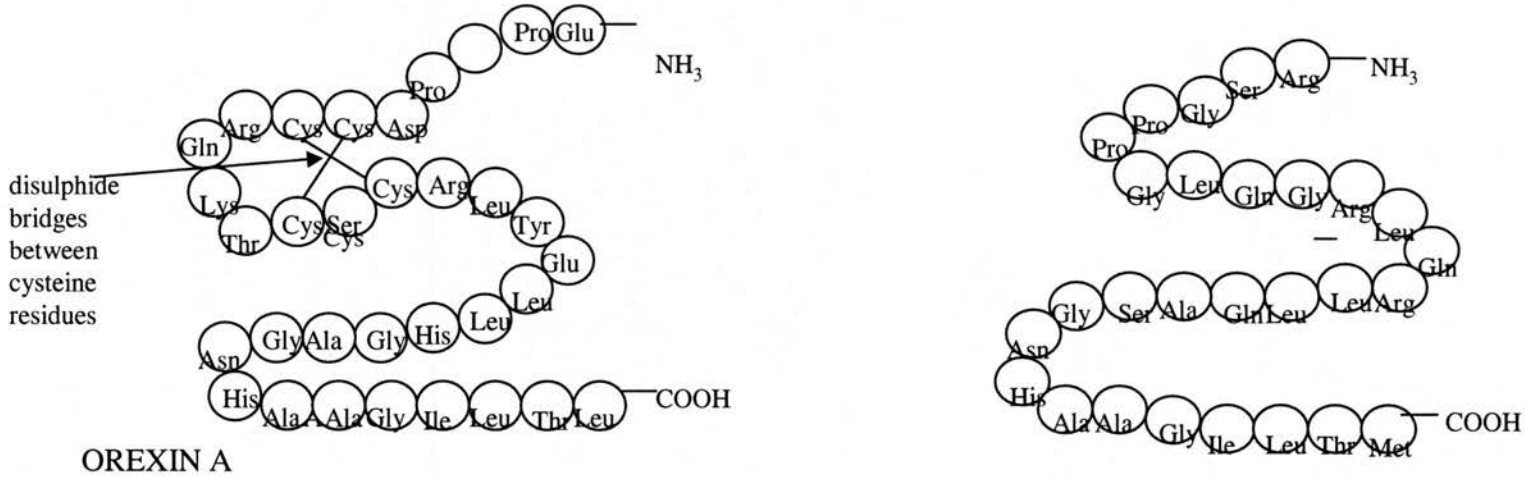


Fig 5.1: Post-translational processing of pp-orexin and the structure of orexin-A and orexin-B.

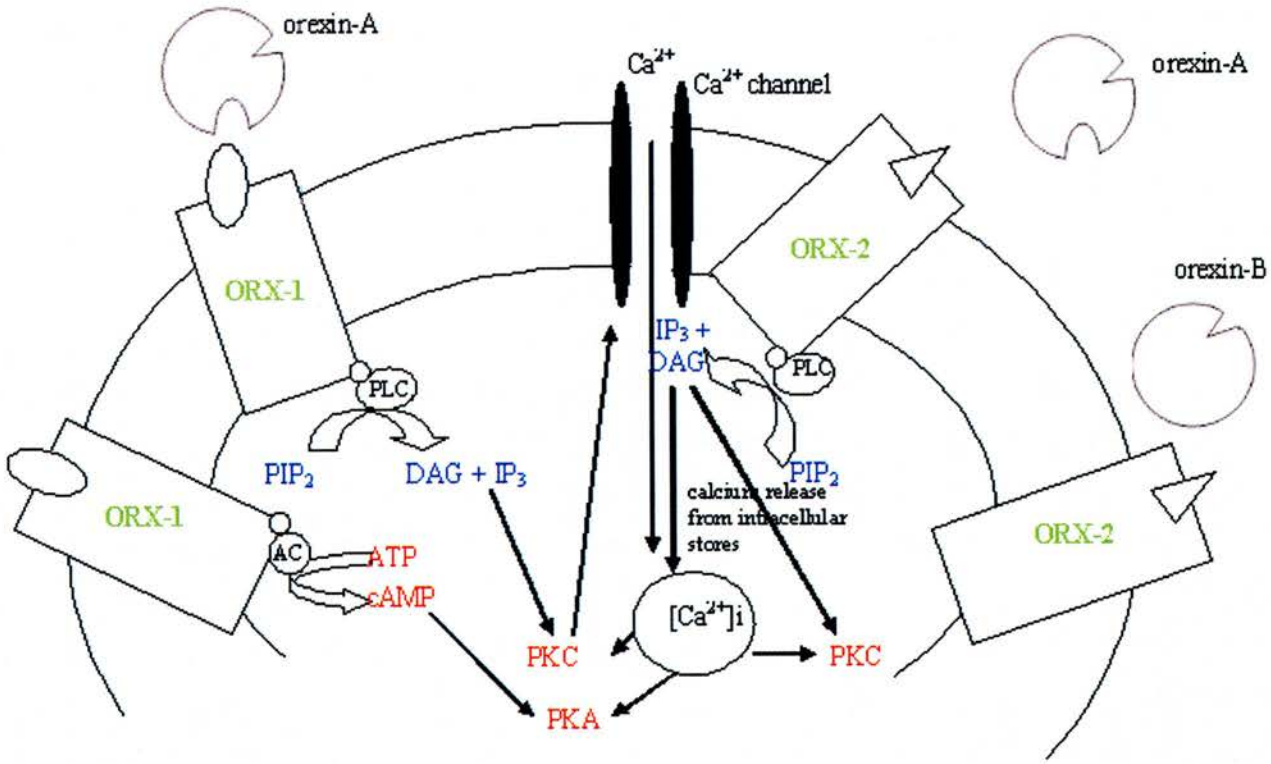


Fig 5.2: Signalling pathways involved in the activation of the orexin receptor

via the IP3 pathway orexin receptor binding also activates the adenylate cyclise signalling pathway.

5.1.4 Expression of the orexins and their receptors in relation to the HPA axis

The orexins receptors were originally discovered in the rat brain. Pre-pro orexin mRNA has been mainly found in the lateral hypothalamic area (LHA) (Lopez *et al,* 2000). Immunohistochemistry showed the orexin positive neurones project widely throughout the brain, including to the arcuate nucleus (ARC), supraoptic nucleus (SON) and paraventricular nucleus (PVN) (Chen *et al,* 1999). The LHA has been importantly implicated in the regulation of food intake; fasting and IIH increased pp-orexin mRNA expression (Cai *et al,* 2001).

OX1-R expression has been shown in the VMH and DMH whereas OX2-R expression has been shown in the LHA, PVN, ARC, mammillary and tuberomammillary nuclei (Trivedi *et al,* 1998). Food deprivation also increases the expression of OX1-R and OX2-R (Karteris *et al,* 2005).

OX1-R and 2 mRNAs have been detected in the pituitary gland (Date *et al,* 2000) whereas no orexin mRNA or protein has been found (Arihara *et al,* 2000).

5.1.5 The effects of orexin on the HPA axis

The PVN contains orexin positive nerve terminals and also orexin receptors (Zhang *et al,* 2005). Central administration of orexin-A increases *c-fos* mRNA expression in the pPVN (Kuru *et al,* 2000) and CRH mRNA in the pPVN (Brunton *et al,* 2003). Orexin-A also stimulates CRH release from hypothalamic explants (Russell *et al,* 2001). Administration of orexin-A depolarises PVN neurones in hypothalamic slices

(Samson *et al,* 2002). Orexin-A stimulation of CRH release is thought to be partly mediated by NPY; orexin increases both CRH and NPY from hypothalamic explants (Russell *et al,* 2001), and increased corticosterone release normally seen after central administration of orexin-A was not seen after pre-treatment with a NPY antagonist (Jaszberenyi *et al,* 2001).

Central administration of orexin-A increases release of ACTH (Brunton *et al,* 2003), but it does not affect basal ACTH release from rat pituitary cells (Samson *et al,* 2001). Orexin-A does not affect either basal or CRH-stimulated cAMP production, so it is suggested the effect of the orexins on the anterior pituitary are mediated by OX1-R coupled to the PKC cascade (Samson *et al,* 2001).

5.1.6 The effect of orexin-A on the HPA axis in pregnancy

Central administration of orexin-A stimulates the HPA axis in virgin female rats (Brunton *et al,* 2003). I.c.v. orexin-A increases plasma ACTH, corticosterone and pPVN CRH mRNA expression in virgin but not pregnant rats (Brunton *et al,* 2003). It is thought that the reduced responsiveness of the HPA axis could be due to increased action of inhibitory inputs to the CRH neurones. Orexin-A may act centrally directly on the pPVN CRH/AVP neurones (Brunton *et al,* 2003). The PVN receives input directly from the LHA (Cuter *et al,* 1999). However, as discussed orexin-A activates the HPA axis directly and indirectly through the NPY neurones since administration of an NPY antagonist inhibits the corticosterone response to orexin-A (Jaszberenyi *et al,* 2001). NPY has also been shown to activate the HPA axis (Haas *et al,* 1989). Thus orexin-A may activate the HPA axis by acting either via the ARC NPY neurones or directly on the PVN (Samson *et al,* 2002).

It is not known what changes occur during pregnancy that lead to reduced HPA axis responses to orexin-A in pregnancy; enhanced inhibitory inputs to the CRH neurones are a possibility (Brunton *et al,* 2003); changes in orexin receptor expression during pregnancy are not known. No differences in either eating, drinking or grooming responses to i.c.v. orexin-A were found between virgin and pregnant rats, so the loss of stimulation of the HPA axis in late pregnancy is not due to a general loss of central responses to orexin-A (Brunton *et al,* 2003).

5.1.7 Aims of experiments

The reduced responsiveness of the hypothalamic CRH-expressing pPVN neurones to centrally administered orexin-A in late pregnancy could be a result of reduced excitatory drive by inputs on which either orexin or NPY have a stimulatory action. There is no reduced capacity to secrete CRH or ACTH in late pregnancy (Ma *et al,* 2005). It has previously been shown that enhanced negative feedback inhibition by glucocorticoids does not underlie the reduced responsiveness of the HPA axis to stress at the end of pregnancy (Johnstone *et al,* 2000). Since NPY may mediate the effect of orexin-A on the HPA axis, reduced HPA axis responses to orexin-A in late pregnancy (Brunton *et al*, 2003) could be a result of the failure of the CRH neurones to respond to NPY (see chapter 6). Endogenous opioid mechanisms have been shown to inhibit SON oxytocin neurones in late pregnancy (Douglas *et al,* 1993). NTS neurones projecting to the PVN may be the source of this opioid. Pro-enkephalin A mRNA expression is upregulated in the NTS in late pregnancy (Brunton *et al*, 2005). It was not known if in pregnancy opioids would interfere with orexin or NPY signalling to the pPVN CRH/AVP neurones. The main aims of these experiments

were: to investigate changes in excitation in hypothalamic circuitry regulating HPA axis and eating responses to centrally administered orexin-A between virgin and pregnant rats and to investigate if endogenous opioids interfere with orexin signalling to the pPVN in late pregnancy.

5.2 Methods

5.2.1 Animals

Female Sprague Dawley rats were used and housed individually after surgery. Rats were maintained as described in section 2.1.

5.2.2 Surgery

Rats were implanted with an intracerebroventricular cannula in experiment 1 and an intracerebroventricular and jugular vein cannula in experiment 2. Surgery was performed under conditions described in section 2.3.

5.2.3 Experiment 1 – The effect of centrally administered Orexin-A in pregnancy

On the day of the experiment (day 21 of pregnancy), rats were left undisturbed for 90 minutes after cannula connection. Rats were given either 5μg orexin-A (5μg in 2μl) or vehicle (artificial cerebro-spinal fluid, aCSF; pH 7.2, composition in mM: NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; NaH_2PO_4, 0.49; $CaCl_2$, 1.26; $MgCl_2$, 1.18) which was given i.c.v. with gentle restraint over a period of about 30 seconds. The following behaviours were noted continually: inactive, grooming, oral motor activity, eating and drinking. Behavioural data were collected for 90 minutes; an event was classified as occurring if an animal spent $\geq$ 5 seconds exhibiting the behaviour. Rats were killed by transcardial perfusion of fixative (4% PFA) under deep pentobarbitone anaesthesia 90 minutes after orexin-A (this time point has previously been shown as the optimum time for showing increased Fos expression in the central nervous system after orexin-A) (Niimi *et al,* 2001). Rats

were examined post-mortem to check presence and number of fetuses (a lower limit of 4 was not counted in the experiment) and to check i.c.v. cannula placement. Brains were processed for Fos immunohistochemistry using free-floating sections and Fos expression measured by counting numbers of neurones with Fos immunoreactive nuclei in regions of interest.

5.2.4 Experiment 2 – The effect of naloxone on reduced HPA responses to orexin-A in pregnancy, on blood glucose and eating behaviour.

On the day of the experiment (day 21 of pregnancy) the cannulae were connected between 07:30-08:30h. The jugular vein cannula was attached to PVC extension tubing led out of the cage and connected to a 1ml syringe filled with heparinised saline (0.9% saline, 50 units/ml). Rats were left undisturbed for 90 minutes and a basal blood sample (0.55ml) was taken. Immediately after the first basal blood sample either naloxone (10mg/ml) or saline both at 0.5ml/kg were given i.v. 15 minutes after the first basal blood sample, another basal blood sample was taken and 15 minutes later rats were given either 5µg orexin-A (5µg/2µl) or vehicle (artificial cerebro-spinal fluid, aCSF; pH 7.2, (composition see 5.2.3) i.c.v. with gentle restraint over a period of about 30 seconds. Further blood samples (0.55ml) were taken for glucose and for ACTH assay at 15, 30, 60 and 120 minutes after i.c.v. infusion. Glucose measurements were made throughout the experiment using a Roche Accuchek Active Meter. Blood samples were placed into eppendorf tubes containing 50µl of chilled 5% EDTA. After each blood sample, blood was replaced with 0.9% sterile saline. Plasma was separated by centrifugation and stored at -20°C until radioimmunoassay. The following behaviours were noted continually: inactive,

grooming, oral motor activity, eating and drinking. Behavioural data were collected for 90 minutes; an event was classified as occurring if an animal spent $\geq$ 5 seconds exhibiting the behaviour. Rats were killed by conscious decapitation 4 hours after orexin-A (this time point has previously been shown as the optimum for showing increased CRH mRNA expression in the PVN following stress) (Harbuz *et al,* 1989). Rats were examined post-mortem to check fetuses and to check i.c.v. cannula placement. Blood samples were processed for ACTH assay and brains processed for CRH and AVP *in-situ* hybridisation.

5.2.5 Fos immunohistochemistry

Coronal sections (52μm) of paraformaldehyde-fixed brains were cut through the PVN, ARC, LHA, VMH and DMH on a freezing microtome onto gelatinised slides. Fos immunohistochemistry was performed using the method described in section 2.5.2. for free floating sections. Fos-positive stained cells were counted in each region with at least 3 sections evaluated per rat. Regions of interest were defined by reference to a rat brain atlas (Konig *et al,* 1963). Cells were counted under x40 magnification on a Leica light microscope.

5.2.6 *In Situ* Hybridisation

Brains were cryostat sectioned at 15μm and mounted onto Polysine slides (BDH) (see section 2.4.2). To detect CRH mRNA expression a 42-mer oligonucleotide probe (MWG-Biotech) was used complementary to bases 496-537, which encode amino acids 22-35 of the rat CRH peptide (Jingami *et al,* 1985). The probe sequence used was:

5'-CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GCC- 3'

The probe was labeled with ^{35}S as described in section 2.4.2. The melting temperature for CRH was 78°C, therefore the heated SSC washes were performed at 58°C. Once dry the sections were dipped in photographic emulsion under safelight conditions and stored at 4°C. The exposure time was 13 weeks for CRH mRNA. The number of positive cells and the silver grain density were measured with the image analysis system described in 2.4.2.

5.2.7 ACTH radioimmunoassay

Plasma ACTH concentration was determined using a commercially available kit as described in section 2.7.3. The sensitivity of the ACTH assay was 6pg/ml. The intra-assay variation was <11%.

5.2.8 Statistics

A two-way analysis of variance (ANOVA) was used with a repeated measures ANOVA for blood sampling experiments. The ANOVA was followed, if it showed significant differences, by Student Newman Keuls multiple comparison tests to identify significant differences between groups. A p value of less than 0.05 was considered statistically significant.

Results

5.3.1 Experiment 1 – The effect of centrally administered orexin-A

Behavioural Data

Analysis of behavioural data using a two-way ANOVA showed a statistically significant difference in eating behaviour following i.c.v. orexin administration (P=0.001). Orexin-A significantly increased eating behaviour in virgin rats (p=0.005; two-way ANOVA) (Fig 5.3). There were no significant differences in drinking behaviour between groups (Fig 5.3). There was a statistically significant increase in grooming behaviour following orexin administration ($p<0.001$) (Fig 5.3). Orexin-A significantly increased grooming behaviour in virgin rats ($p<0.001$; two-way ANOVA) (Fig 5.3). Occurrence of eating and grooming in late pregnant rats after i.c.v. orexin were not significantly different from their occurrence in virgin rats; although in pregnant rats eating and grooming were not significantly increased, there was a trend for both eating and grooming to increase following administration of orexin.

Parvocellular Paraventricular Nucleus (pPVN) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups (p=0.002). Quantification of Fos positive cells revealed that Fos expression in the PVN of the virgin orexin-treated group was significantly greater (2-fold) than in the pregnant orexin group (Fig 5.4). I.c.v. injection of Orexin significantly increased Fos expression in the pPVN in the virgin group ($p<0.001$) (21.6 ± 1.4 vs control 10.5 ± 2.1 Fos positive cells) (Fig 5.4 & Fig 5.5).

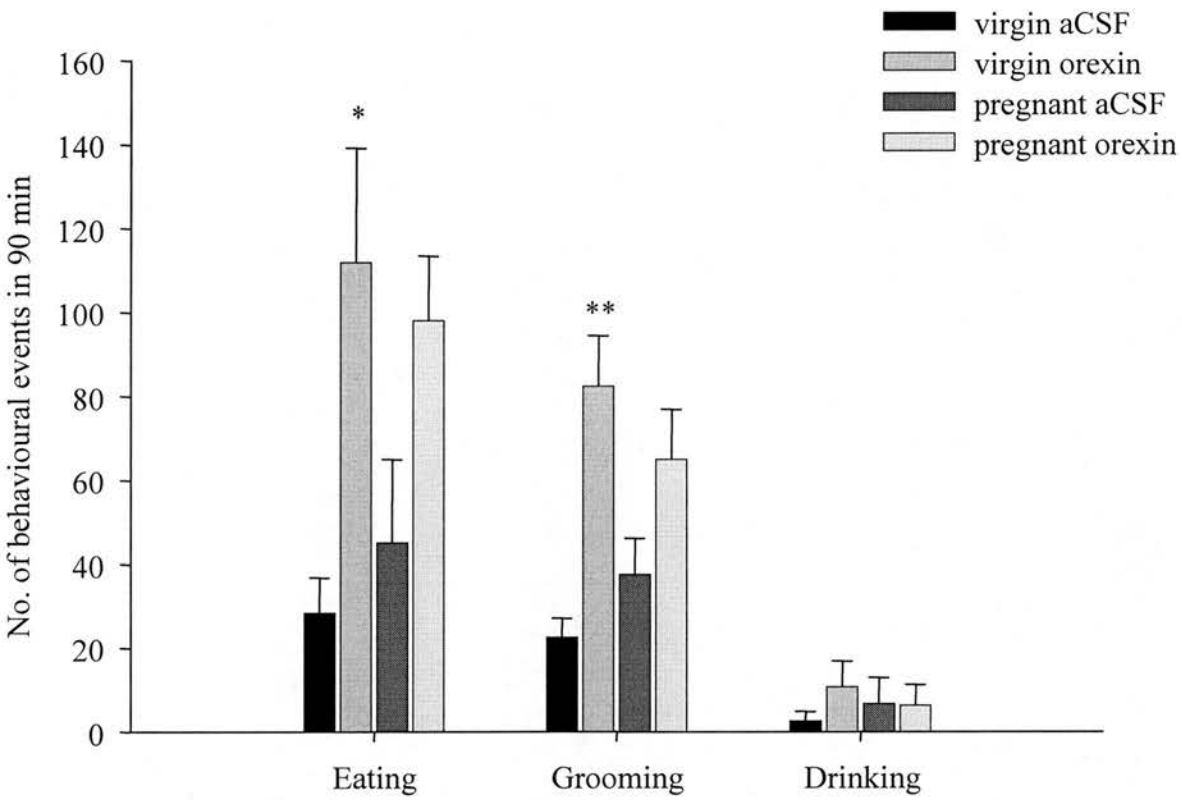


Figure 5.3: The effect of i.c.v. orexin-A on behvaiours in virgin and pregnant rats. Behaviours were monitored ontinually for 90 minutes post i.c.v. injection of orexin-A (5μg/rat). An event was classified as occuring if an animal spent > seconds exhibiting the behaviour. Data represent group means ± SEM. Virgin/ aCSF n=7; virgin/orexin, n=8; pregnant/aCSF, n=7; pregnant/orexin, n=7. **p<0.001 vs aCSF, *p<0.005 vs aCSF; two-way ANOVA followed by Student Newman Keuls multiple comparison tests.

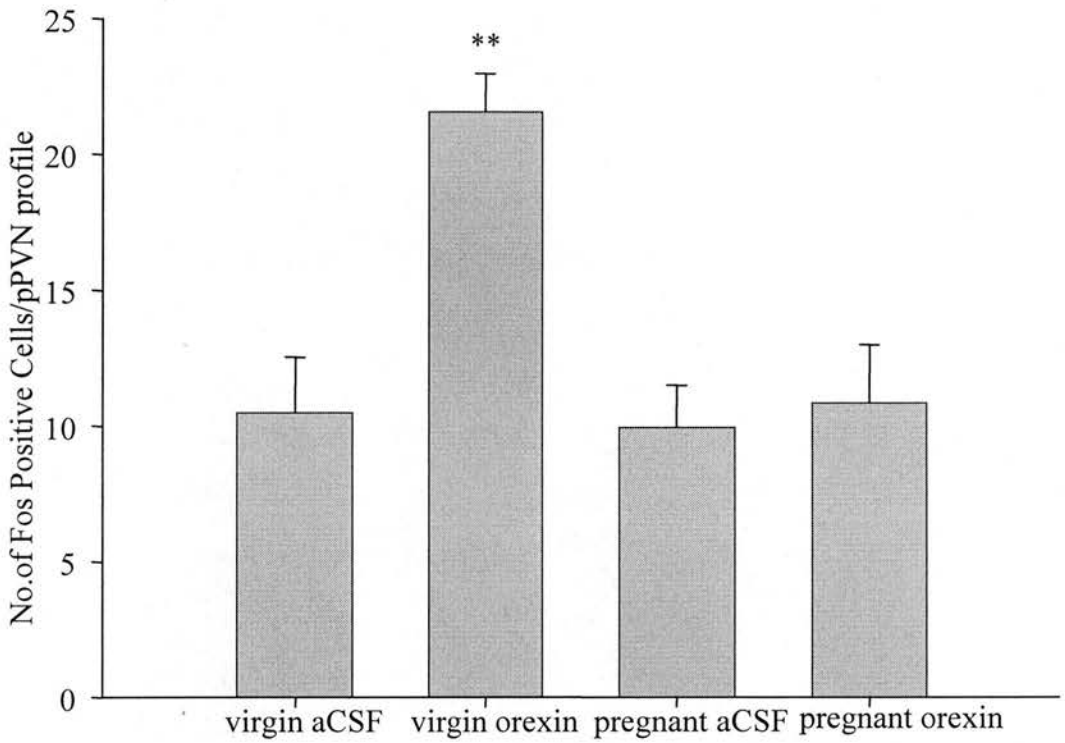


Figure 5.4: The effect of i.c.v. orexin-A on Fos expression in the pPVN in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 minutes post-Injection of orexin-A. Values are the mean count positive cells over three sections per rat and values are the group means ± SEM. Virgin.aCSF, n=7; virgin/orexin, n=8; pregnant/aCSF, n=7; pregnant/orexin, n=7. Two-way ANOVA followed by Student Newman Keuls multiple comprison tests. **$p<0.001$ vs all other groups.

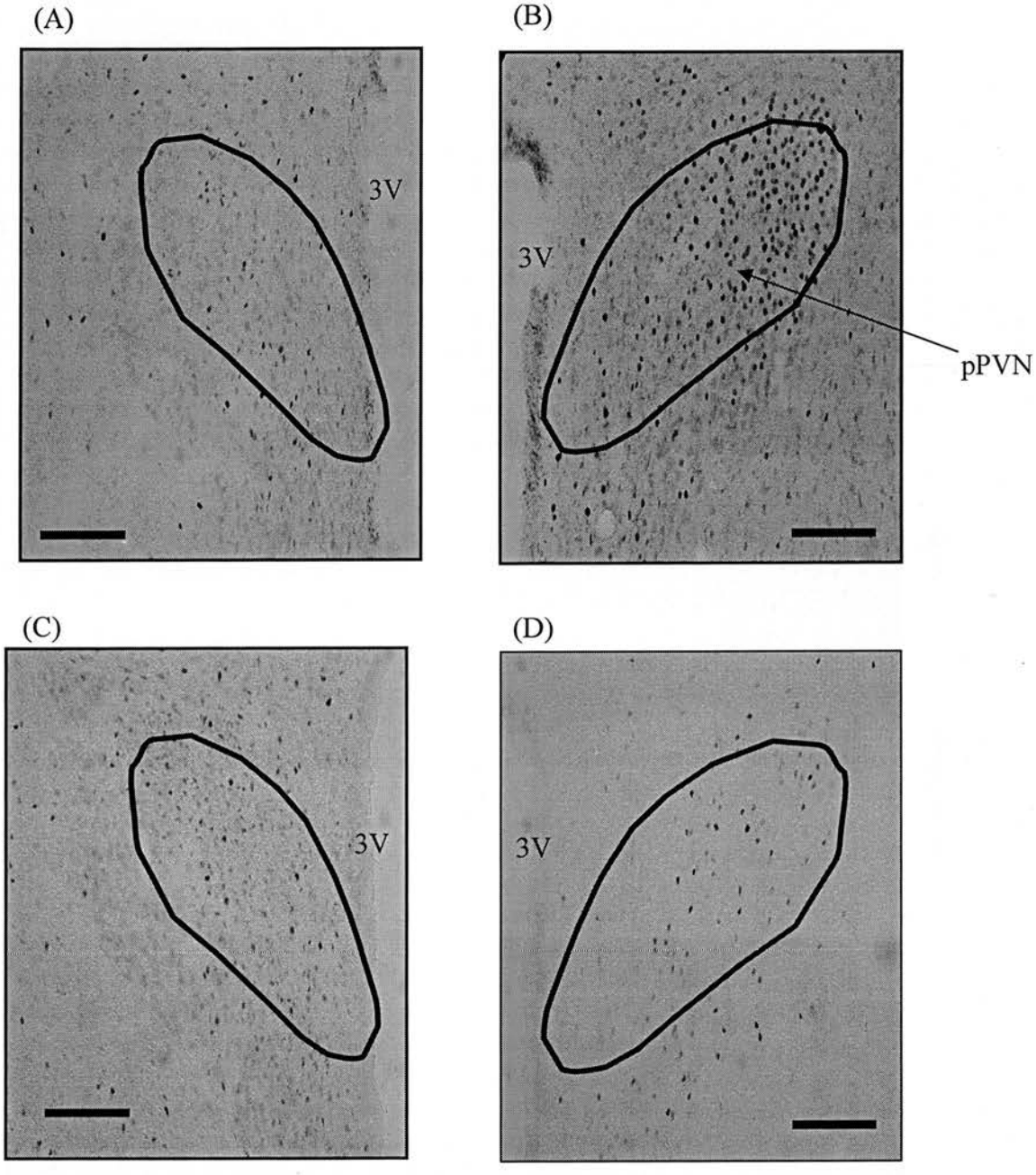


Figure 5.5: Representative photomicrographs of microtome sections through the pPVN of microtomal sections (52µm) in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry for (A) virgin aCSF; (B) virgin orexin-A; (C) pregnant aCSF; (D) pregnant orexin-A treated rats. Scale bar = 100µm. 3V = 3rd ventricle. pPVN = parvocellular PVN.

Arcuate nucleus (ARC) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among the virgin and pregnant groups ($p<0.001$). Quantification of Fos positive cells revealed that Fos expression in the ARC of virgin rats was significantly greater (3-fold) than in the pregnant orexin group (Fig 5.6). I.c.v. injection of orexin significantly increased Fos expression in the virgin group ($p<0.001$) (27.6 ± 2.9 vs control 9.6 ± 1.5 Fos positive cells/ARC profile) (Fig 5.6 and Fig 5.7).

Lateral Hypothalamic Area (LHA) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among the virgin and pregnant groups ($p=0.017$). Quantification of Fos positive cells revealed that Fos expression in the LHA of pregnant rats was significantly greater (1.4-fold) than in the virgin orexin group (Fig 5.8). I.c.v. injection of orexin significantly increased Fos expression in the virgin group ($p=0.006$) (19.9 ± 3.0 vs control 11.1 ± 1.2 Fos positive cells/LHA profile) (Fig 5.8) and in the pregnant group ($p<0.001$) (27.6 ± 2.3 vs 13.9 ± 1.6 Fos positive cells/LHA profile) (Fig 5.8 and Fig 5.9).

Ventromedial Hypothalamus (VMH) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among the virgin and pregnant groups ($p=0.0033$) (Fig 5.10). Quantification of the number of Fos positive cells revealed that Fos expression in the VMH of pregnant rats given orexin-A was significantly greater (1.5 times) than in the virgin orexin group (Fig 5.10). I.c.v. injection of orexin-A significantly increased Fos expression in the pregnant group

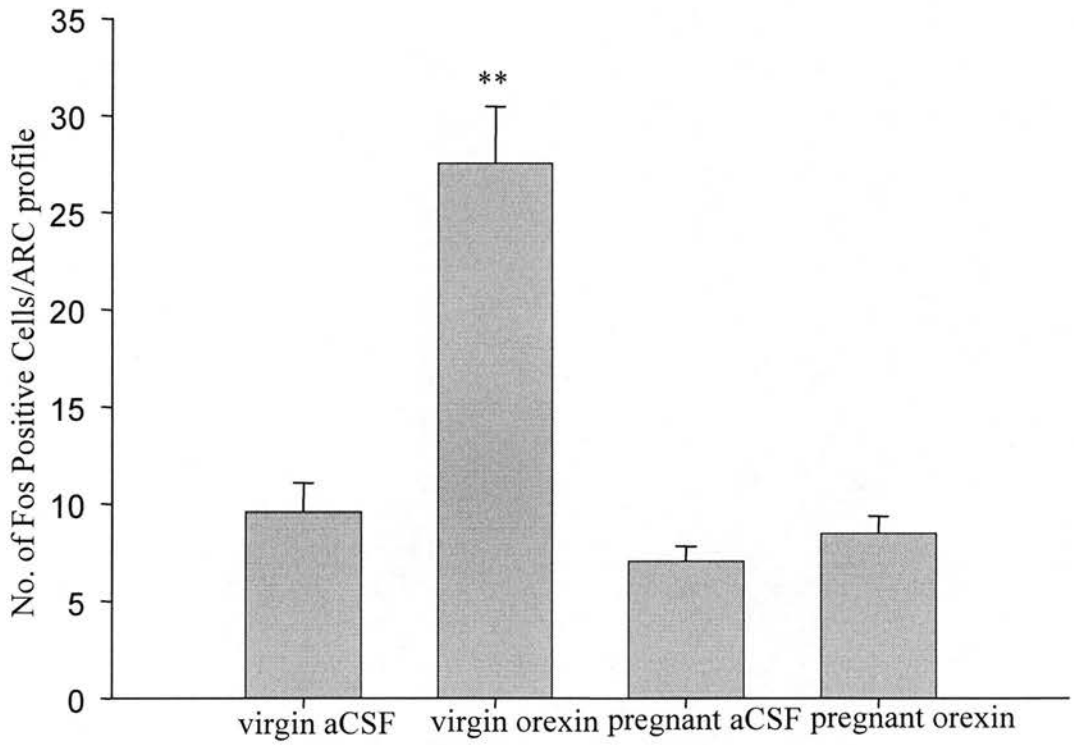


Figure 5.6: The effect of i.cv. orexin-A on Fos expression in the ARC in virgin and pregnant rats. Rats were killed by transcardial fixation perfusion 90 minutes Post-injection of orexin-A. Values are the mean counts of postiive cells over three sections per rat and are shown as the group means ± SEM. Virgin/aCSF, n=7; virgin/orexin, n=8; pregnant/aCSF, n=7; pregnant/orexin, n=7. Two-way ANOVA followed by Student Newman Keuls multiple comparison tests. **$p<0.001$ vs all other groups.

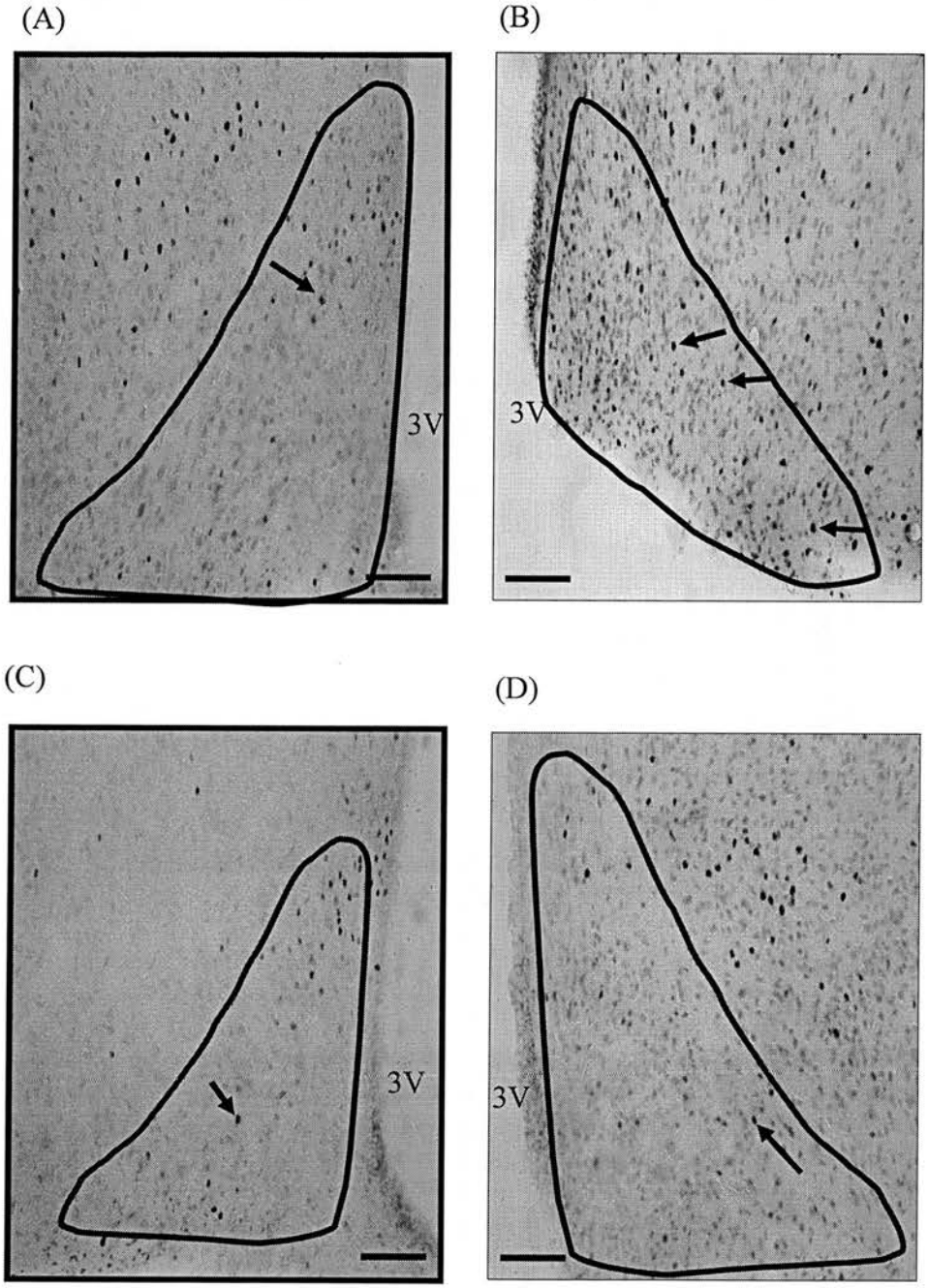


Figure 5.7: Representative photomicrographs of coronal sections through the ARC of microtome sections (52µm) in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry groups: (A) virgin aCSF; (B) virgin orexin-A; (C) pregnant aCSF; (D) pregnant orexin-A. Small arrows indicate Fos-positive nuclei; the solid line outlines the arcuate nucleus. Scale bar = 100µm. 3V = 3rd ventricle.

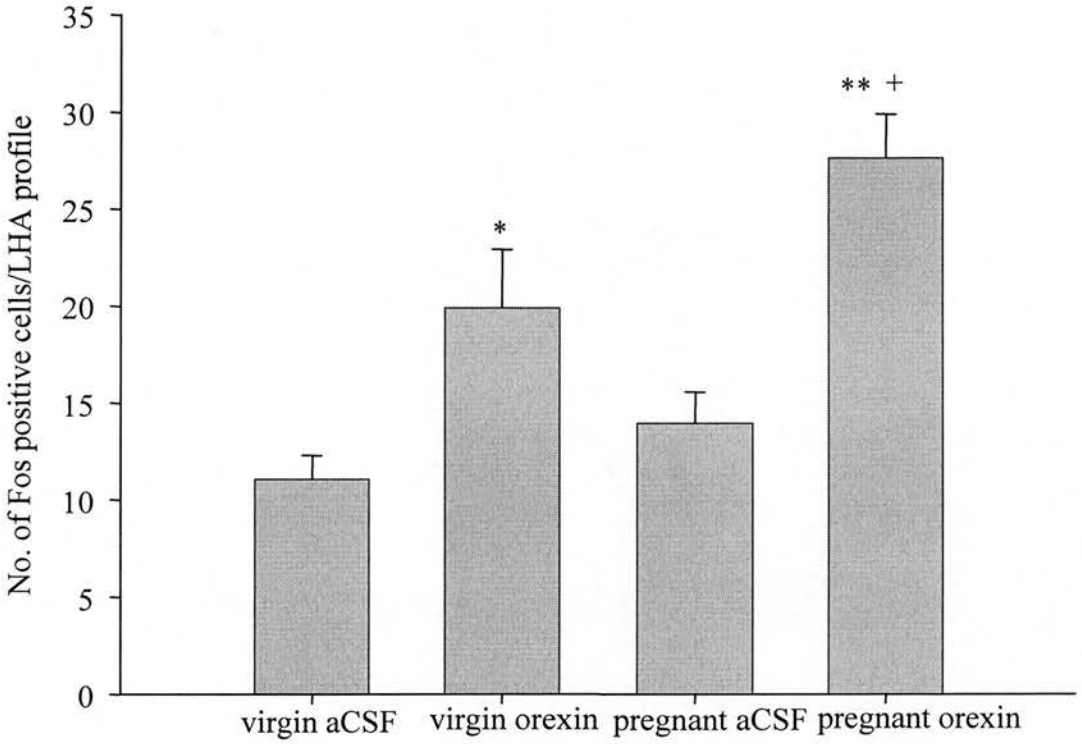


Figure 5.8: The effect of i.c.v. orexin-A on Fos counts in the LHA in virgin and pregnant rats. Rats were killed by transcardial fixation perfusion 90 minutes post-injection of orexin. Values are the mean count positive cells over three sections per rat and are shown as the group means ± SEM. Virgin/aCSF, n=7; virgin/orexin, n= 8; pregnant/aCSF, n=7; pregnant/orexin, n=7. Two-way ANOVA followed by Student

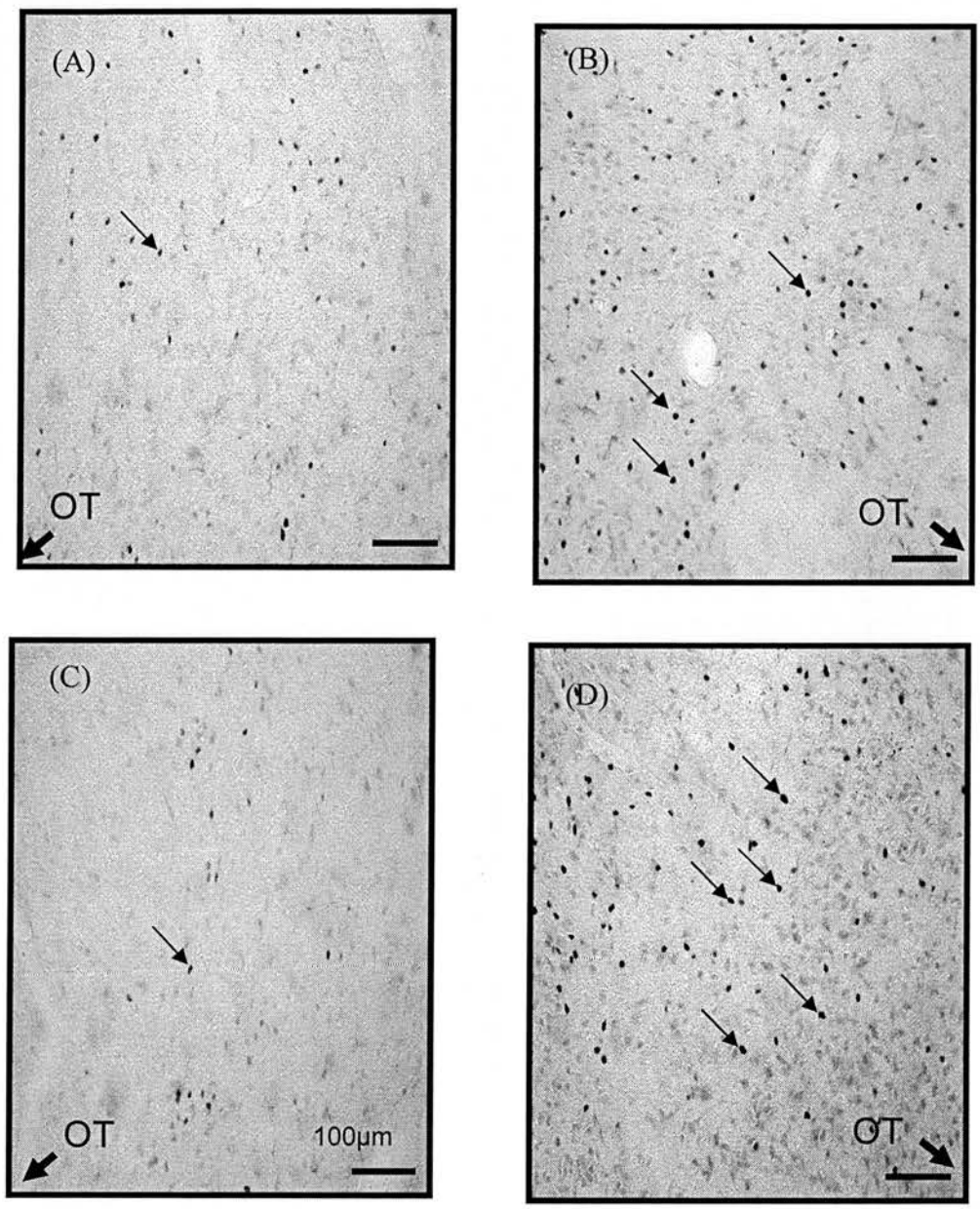


Figure 5.9: Representative photomicrographs of coronal sections through the LHA of microtome sections (52µm) in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry groups: (A) virgin aCSF; (B) virgin orexin-A; (C) pregnant aCSF; (D) pregnant orexin-A. Scale bar = 100µm. OT = optic tract.

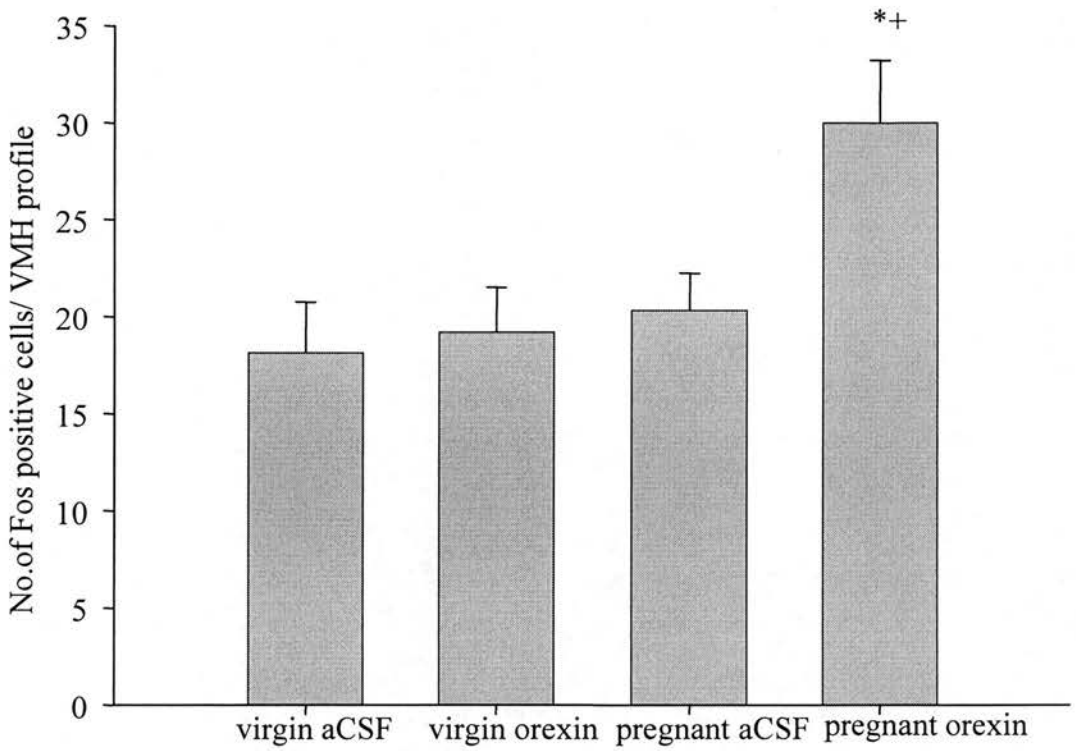


Figure 5.10: The effect of i.c.v. orexin-A on Fos counts in the VMH in virgin and pregnant rats. Rats were killed by transcardial perfusion 90 minutes post-injection of orexin-A. Values are the mean count positive cells over three sections per rat and are shown as group means ± SEM. Virgin/aCSF, n=7; virgin/orexin, n=8; pregnant/ aCSF, n=7; pregnant/orexin, n=7. Two-way ANOVA followed by Student Newman Keuls multiple comparison tests. *$p<0.05$ vs virgin orexin; +$p<0.003$ vs aCSF.

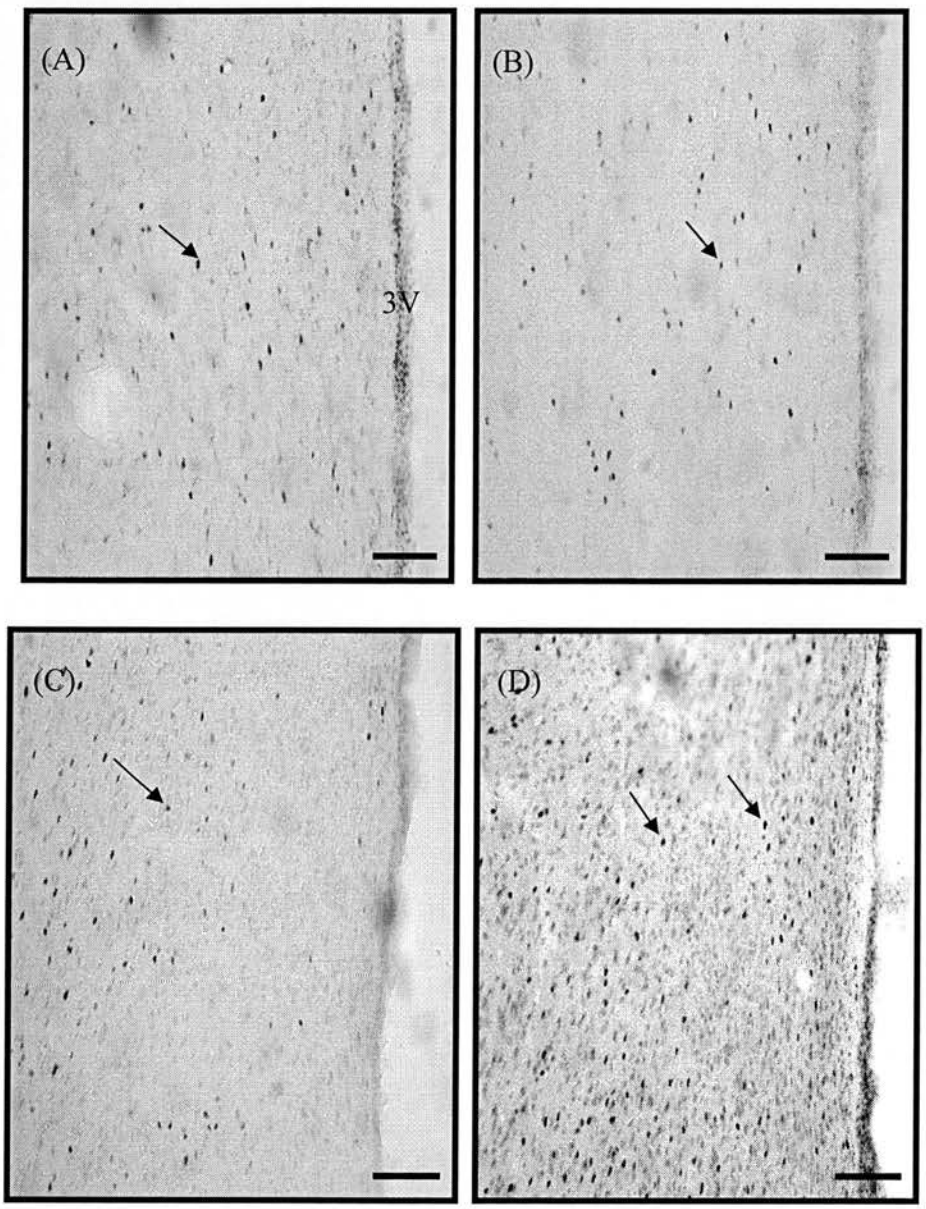


Figure 5.11: Representative photomicrographs of coronal sections through the VMH of microtome sections (52μm) in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry groups: (A) virgin aCSF; (B) virgin orexin-A; (C) pregnant aCSF; (D) pregnant orexin-A. Scale bar = 100μm. 3V = 3rd ventricle

($p=0.003$) (30.0 ± 3.2 vs control 20.3 ± 1.9 Fos positive cells/VMH profile) but not in virgin rats (Fig 5.10 and Fig 5.11).

Dorsomedial Hypothalamus (DMH) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was not a statistically significant difference in Fos expression among the virgin and pregnant groups (Fig 5.12). I.c.v. injection of orexin did not significantly increase Fos expression in either the virgin or the pregnant group (Fig 5.12).

6.3.2 Experiment 2 – The effect of naloxone on reduced HPA responses to orexin-A in pregnancy, and on blood glucose and eating behaviour.

Behavioural data

Analysis of behavioural data using a two-way ANOVA showed there was no statistically significant difference in eating behaviour among groups (Fig 5.13). Also there were no significant differences in drinking or grooming between groups (Fig 5.13).

Blood glucose concentration

Analysis of blood glucose concentration using a two-way RM ANOVA showed a statistically significant difference in plasma glucose concentration among virgin and pregnant groups ($p<0.001$). Basal blood glucose concentrations were significantly lower in pregnant rats overall than virgins ($p<0.05$; two-way RM ANOVA). Orexin with i.v. saline significantly increased blood glucose in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min (7.4 ± 0.2 vs 5.7 ± 0.4 mmol/l) with glucose concentrations returning to the post-naloxone basal value by 30 min (Fig 5.14). I.c.v.

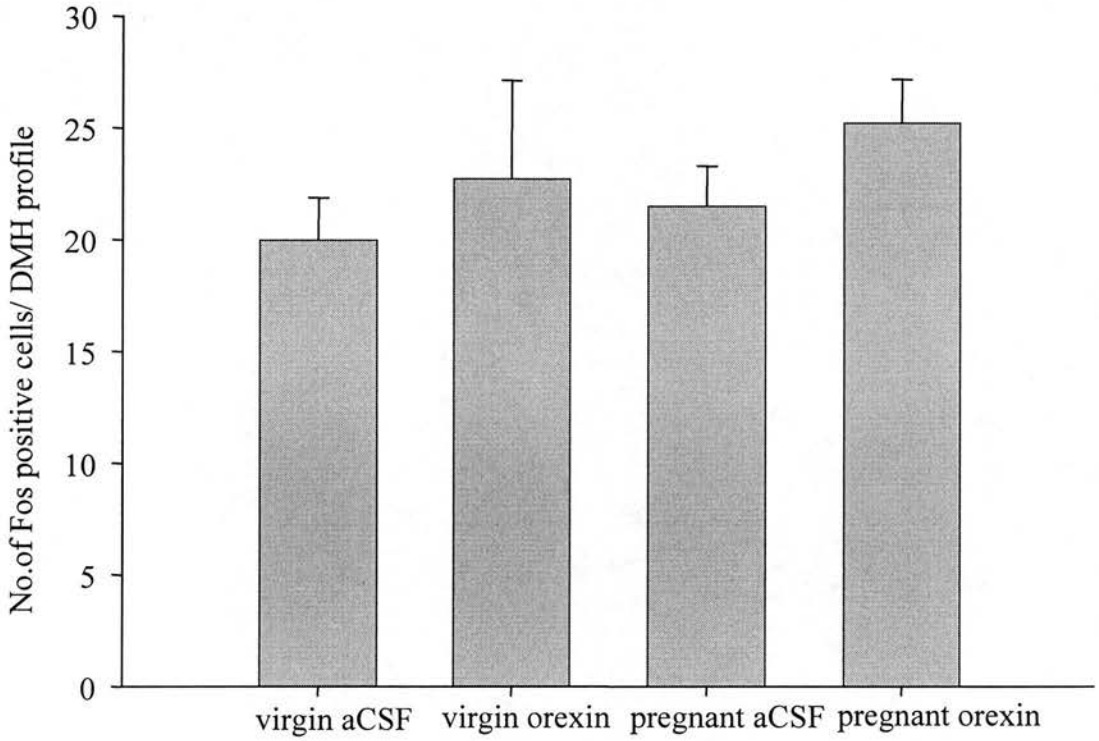


Figure 5.12: The effect of i.c.v. orexin-A on Fos counts in the DMH in virgin and pregnant rats. Rats were killed by transcardial fixation 90 minutes post-injection of orexin. Values are the mean count positive cells over three sections per rats and shown as group means ± SEM. Virgin/aCSF, n=7; virgin/orexin, n=8; pregnant/ aCSF, n=7; pregnant/orexin, n=7. A two-way ANOVA showed no significant Differences.

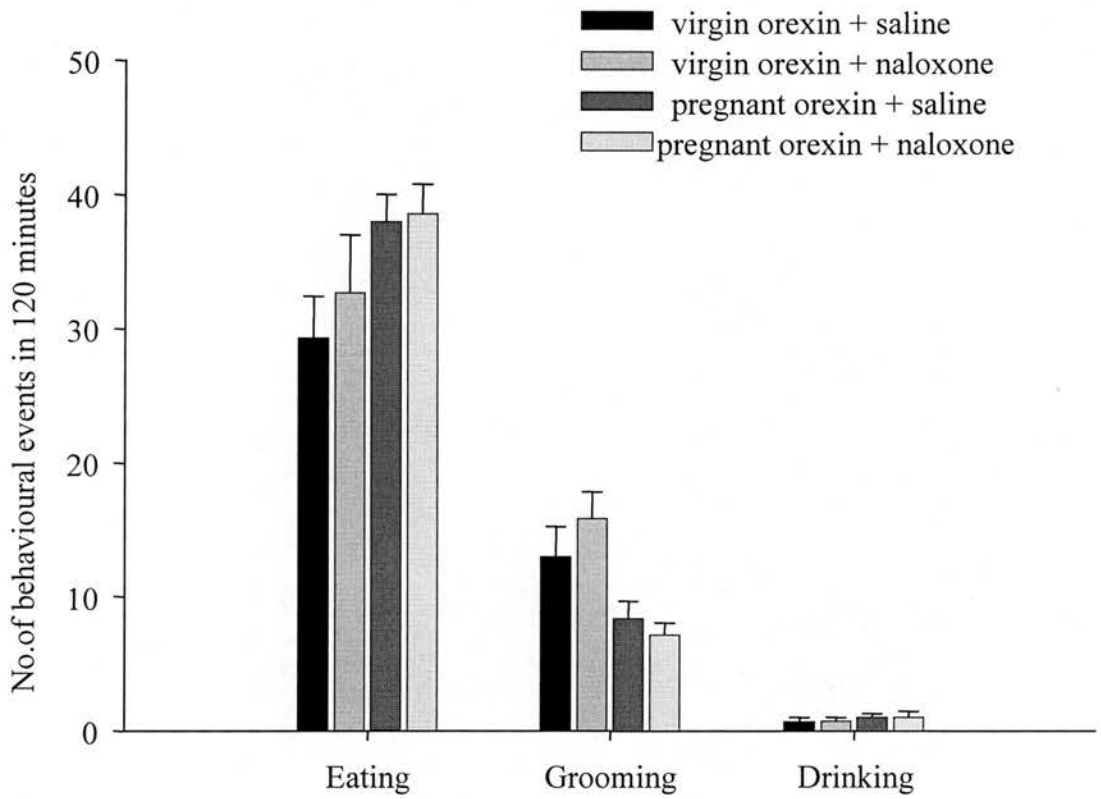


Figure 5.13: The effect of i.c.v. orexin-A and i.v. naloxone on behaviours in virgin and pregnant rats. Behaviours were monitored continually for 120 minutes post-injection of orexin-A (5μg/rat). An event was classified as occurring if an animal spent 5 seconds exhibiting the behaviour. Data represent group means ± SEM. Virgin orexin/saline, n=6; virgin orexin/naloxone, n=7; pregnant orexin/saline, n=6; pregnant orexin/naloxone, n=7. Two-way ANOVA showed no signficant differences Among groups.

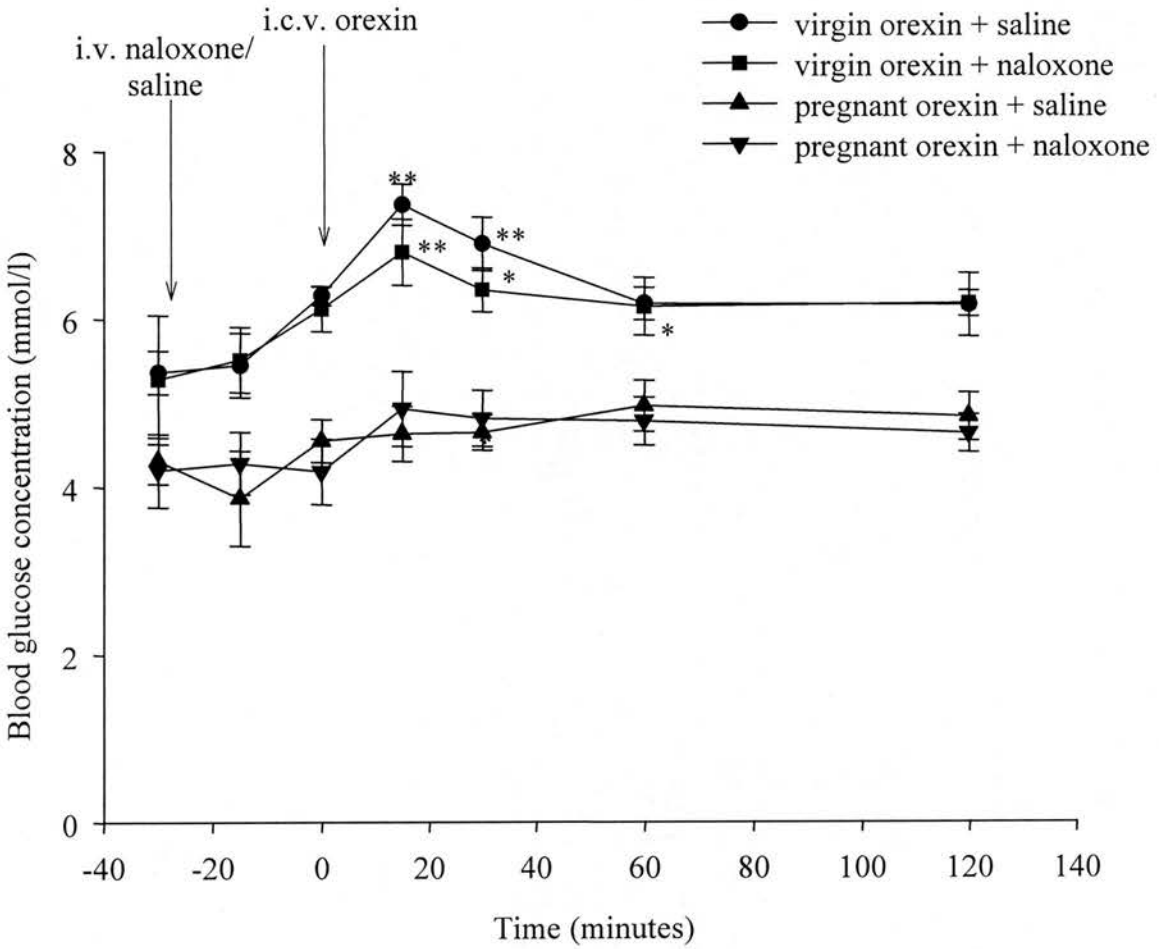


Figure 5.14:The effect of i.c.v. orexin-A and i.v. naloxone on blood glucose concentration in virgin and pregnant rats. Three basal blood samples were taken 30, 15 and 1 min prior to i.c.v. orexin (5μg/rat). I.v. naloxone was given 30 minutes prior to i.c.v. orexin. Further blood samples were withdrawn 15, 30, 60 and 120 min post-infusion. Values are group means ± SEM. Virgin orexin/saline, n=6; virgin orexin/naloxone, n=7; pregnant orexin/saline, n=6; pregnant orexin/ Naloxone, n=7. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests: **p<0.001, *p<0.05 significantly different from the 1st

injection of orexin with i.v. naloxone significantly increased blood glucose in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min (6.8 ± 0.4 vs basal 5.6 ± 0.6 mmol/l) with glucose concentrations returning to the post-naloxone basal value by 30 min (Fig 5.14). I.c.v. injection of orexin and i.v. naloxone had no significant effect on blood glucose concentration in pregnant rats, nor did i.c.v. injection of orexin and i.v. saline. Naloxone had no significant effect on the blood glucose response to orexin-A in virgin rats, or the lack of a response in pregnant rats (Fig 5.14).

Plasma ACTH concentration

Analysis of plasma ACTH concentration data using a two-way RM ANOVA showed there was a statistically significant difference in plasma ACTH concentrations among the virgin and pregnant groups ($p = 0.031$). Data are shown as the percentages of the basal values after i.v. naloxone as there was variation between basal values. Basal values were virgin orexin + saline 70.1 ± 4.5 pg/ml; virgin orexin + naloxone 40.3 ± 4.8 pg/ml; pregnant orexin + saline 77.7 ± 4.9 pg/ml; pregnant orexin + naloxone 77.0 ± 3.7 pg/ml. These concentrations however were not significantly different among groups. I.c.v injection of orexin-A and i.v. naloxone significantly increased plasma ACTH concentration in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min (231.3 ± 36.4 vs basal 100%) (Fig 5.15 and Fig. 5.16), with concentrations remaining significantly elevated for 240 min (Fig 5.15). I.c.v. injection of orexin and i.v. saline significantly increased plasma ACTH concentration in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min; (188.4 ± 17.9 vs basal 100%) (Fig 5.15); with concentrations remaining significantly elevated for 15 min (Fig 5.15 and 5.16). I.c.v orexin also significantly increased plasma ACTH in

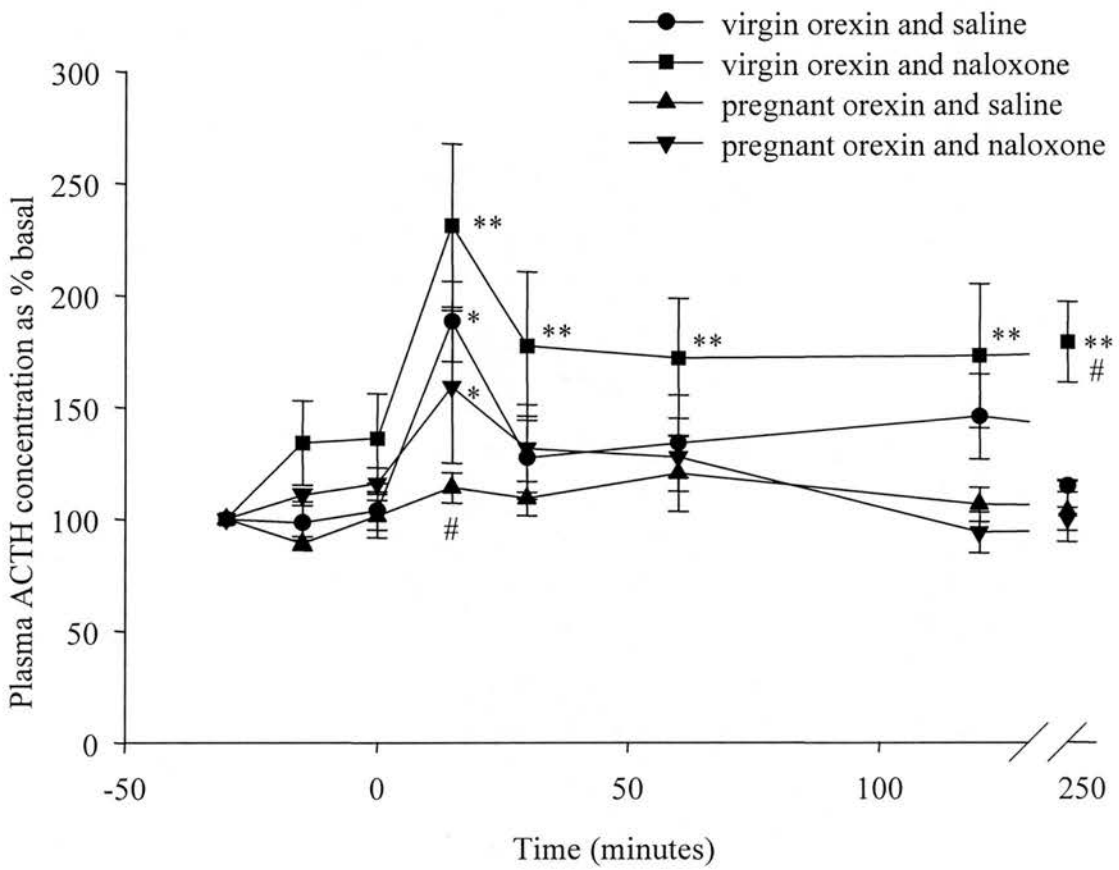


Figure 5.15: The effect of i.c.v. orexin-A and i.v. naloxnoe on plasma ACTH concentration in virgin and pregnant rats. Three basal blood samples were taken 30, 15, and 1 min prior to i.c.v. injection of orexin-A (5µg/rat). Further blood samples were taken 15, 30, 60, 120 and 240 (trunk blood) min post-injection. I.v. naloxone was given 30 min prior to injection of orexin-A. Data represent group means ± SEM. Virgin orexin/saline, n=6; virgin orexin/naloxone, n=7; pregnant orexin/saline, n=6; pregnant orexin/naloxone, n=7. Two-way ANOVA for repeated measures followed by a Student Newman Keuls multiple comparison test: **p<0.001, *p<0.05 vs basal, #p<0.05 vs all other groups at the same time point.

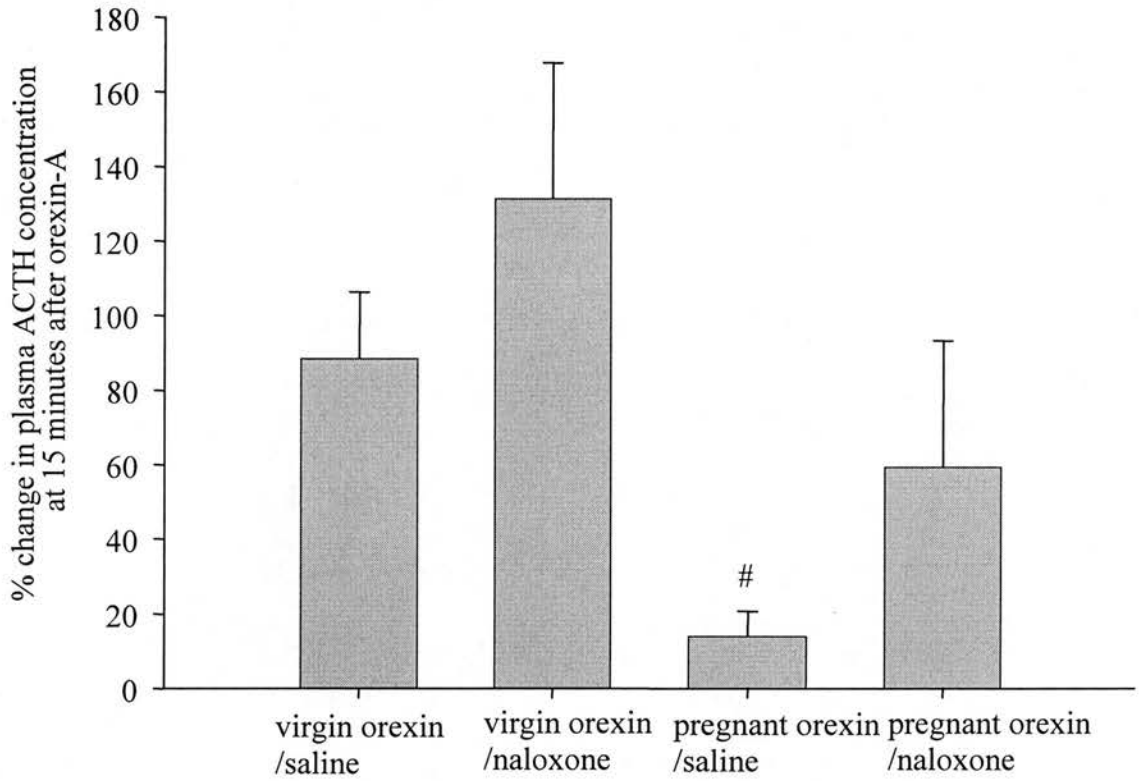


Figure 5.16: The effect of i.c.v. orexin-A and i.v. naloxone on plasma ACTH concentration in virgin and pregnant rats. % delta values for the measurements at 15 minutes after orexin-A (5μg/rat) subtracted from the first basal blood sample were calculated. Values are the group means ± SEM. Vrigin orexin/saline, n=6; virgin orexin/naloxone, n=7; pregnant orexin/saline, n=6; pregnant orexin/naloxone, n=7. A two-way ANOVA followed by a Student Newman Keuls multiple comparison test. #$p<0.05$ vs all other groups.

the pregnant group given i.v. naloxone (p=0.003; two-way RM ANOVA) within 15 min (159.2 ± 34.1 vs basal 100%) (Fig 5.16) with concentrations remaining elevated for 15 minutes (Fig 5.15 and Fig 5.16). I.c.v. orexin given together with i.v. saline did not alter plasma ACTH concentration in late pregnant rats at any time point. Naloxone alone groups were not included in this experiment, however the effect of naloxone alone was investigated on plasma ACTH, CRH and AVP mRNA expression and showed no differences (Brunton unpublished).

Parvocellular PVN (pPVN) CRH mRNA expression

Analysis of CRH mRNA expression using a two-way ANOVA showed there was a statistically significant difference in the number of pPVN neurones expressing CRH mRNA among the virgin and pregnant groups (p=0.002). The number of CRH mRNA expressing neurones in the pPVN was significantly greater (2.5 fold) in the virgin/orexin-A group than in the pregnant/orexin-A group, both treated with saline (Fig 5.17). I.c.v. injection of orexin with i.v. naloxone significantly increased CRH mRNA expression in the pregnant group (p<0.001) compared with i.c.v. injection of orexin and i.v. saline, (Fig 5.17), and expression in the orexin/naloxone treated pregnant rats was not different from expression in either virgin group given orexin-A.

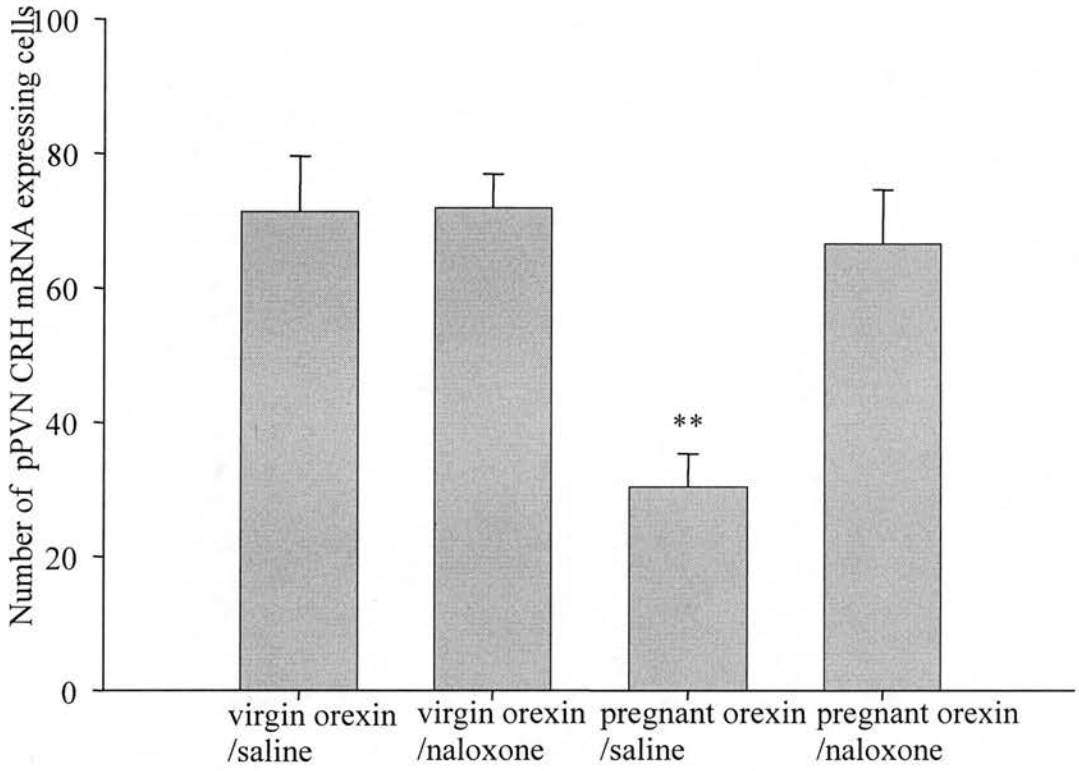


Figure 5.17: The effect of i.c.v. orexin-A on CRH mRNA expression in the parvocellular PVN in virgin and pregnant rats. Rats were killed by conscious decapitation 240 minutes post-injection of orexin-A. Values are the mean counts of positive cells over at least three sections per rats and values are the group means ± SEM. Virgin orexin/saline, n=6; virgin orexin/naloxone, n=7; pregnant orexin/saline, n=6; pregnant orexin/naloxone, n=7. A two-way ANOVA followed by a Student Newman Keuls multiple comparison test. **$p<0.01$ vs all other groups.

5.4 Discussion

5.4.1. The effect of i.c.v. orexin-A and naloxone on behavioural parameters

The results show that centrally administered orexin-A stimulates eating behaviour in both virgin and pregnant rats (Fig 5.3), confirming previous findings (Brunton *et al,* 2003). This has been shown also in male rats (Sakurai et al, 1998). Rats in experiment two seemed to eat more than rats in experiment one, this is due to the effect of different analysers in both experiments. Naloxone did not affect eating in either virgin or pregnant rats given orexin-A. Nonetheless, there is strong evidence that opioid peptides stimulate feeding and elicit satiety, and the stimulatory actions of i.c.v. NPY on eating are inhibited by naloxone (see Chapter 6). Opioids have long been known to play a role in the regulation of ingestive behaviour via the receptor subtypes kappa, mu and delta (Baile *et al,* 1986). Central administration of opioid agonists stimulate food intake; opioid peptides from all three families, endorphins, enkephalins and dynorphins, have been shown to stimulate feeding in various species (Baile *et al,* 1986). Administration of opioid antagonists does the reverse, decreasing food intake (Yu *et al,* 1997). It seems however that orexin-A stimulates feeding behaviour independently of opioid actions.

Orexin-A did not significantly stimulate drinking behaviour in either virgin or pregnant rats. Other studies have shown no significant changes in drinking in response to i.c.v. orexin administration (Brunton *et al,* 2003). However, centrally injected orexins have been found to increase drinking at doses similar to those that elicit increased feeding (Kunii *et al,* 1999). The LHA is a primary drinking centre (Greer *et al,* 1955), and orexin-A might exert any actions on drinking here, while there is orexin innervation of the area postrema a region important in fluid

homeostasis (Kunii *et al,* 1999). The lack of an increase in drinking in the present study after orexin may reflect the weak effect on food intake.

The present results showed that orexin-A significantly increased grooming in virgin rats with a tendency also for grooming to increase in pregnant rats. This has previously been shown in male (Willie *et al,* 2001) and female rats (Brunton *et al,* 2003). The effects of centrally administered orexin-A on grooming could reflect activational actions of orexin in the regulation of feeding and the sleep-wakefulness cycle (Lin *et al,* 1999). The brain region receiving the densest innervation from orexigenic neurones is the locus coeruleus, and orexin-A increases cell firing of noradrenergic neurones (Hagan *et al,* 1999). Grooming has been linked to elevated stress levels (Dunn *et al,* 1981), a suggestion consistent with the observations that orexin-A increases plasma corticosterone levels (Jones *et al,* 2001). Naloxone did not significantly alter drinking or grooming after orexin-A in either virgin or pregnant rats.

5.4.2. Changes in Fos expression in hypothalamic circuitry regulating HPA axis and eating behaviour after centrally administered orexin-A in late pregnancy

pPVN. I.c.v. orexin-A significantly increased the number of Fos positive cells in the pPVN in virgin rats compared with aCSF treated virgin rats (Fig.5.5). There was also a significantly greater number of Fos positive cells in the pPVN of virgin rats compared to late pregnant rats given orexin-A. This suggests there is reduced responsiveness of the CRH/AVP neurones in pregnancy in the pPVN as these neurones comprise a major proportion of the pPVN neurones (91%; Arima *et al,* 2000).

CRH neurones are also involved in reducing food intake. Responsiveness of the CRH neurones other than in the pPVN in pregnancy is not known. Following i.c.v. injection of orexin-A Fos expression was highly expressed in the PVN (Edwards *et al,* 1999). Since many neurones are activated by injection of orexin-A, orexin was injected into the PVN directly (Edwards *et al,* 1999) to determine whether this might be the most sensitive area for the orexigenic effect of orexin-A. Orexin injected into the PVN did not stimulate food intake to the same extent as i.c.v. orexin-A. Thus, orexin-A may not have a direct effect on the PVN (Edwards *et al,* 1999). It is known that the stimulatory action of orexin-A on pPVN CRH neurones is at least partly mediated by NPY, and NPY neurones in the ARC have a major role in regulating appetite (Jain *et al,* 2000). Orexin A increased CRH and NPY release from hypothalamic explants and its stimulatory effect on CRH release was blocked by a Y1 receptor antagonist (Russell *et al,* 2001).

ARC. In the present study centrally administered orexin-A significantly increased the number of Fos positive cells in the ARC in virgin rats compared with aCSF treated rats (Fig 5.7). There was also a significantly greater number of Fos positive cells in the ARC after i.c.v. orexin-A in virgin rats compared to late pregnant rats. This suggests that the lack of activation of ARC neurones in pregnancy does not interfere with the actions of orexin-A in stimulating appetite in pregnancy since pregnant rats showed undiminished eating behaviour responses to i.c.v. orexin. ARC NPY neurones are importantly implicated in the role of orexin in feeding especially via the Y1 receptor (Jain *et al*, 2000). Orexin neurones project to the ARC (Peyron *et al,* 1998) and specifically innervate NPY neurones (Horvath *et al,* 1999). Orexin-stimulated feeding is only partially inhibited by pre-treatment with a selective NPY

Y1 receptor antagonist, suggesting that activation of the NPY-mediated feeding pathway is only partially responsible for orexin-stimulated feeding.

LHA. The present findings indicate that orexin-A can stimulate feeding other than through this mechanism, e.g. by directly acting on the LHA, perhaps stimulating MCH neurons. The LHA receives innervation from much of the neuraxis and can also be influenced by actively transported peripheral factors e.g. leptin, insulin and other hormones, as well as other diffusible factors including glucose, electrolytes, amino acids and peptides (Bernardis & Bellinger, 1996). Therefore the regulation of orexin neurones in the LHA is potentially complex. Orexin neurones in the LHA are activated by hypoglycaemia; insulin increases Fos expression in orexin neurones in the LHA (Moriguchi *et al,* 1999). When insulin and glucose are given together so that euglycaemia was maintained no increase in Fos is found. Insulin is therefore unlikely to act directly on these neurones. Insulin does not increase Fos in MCH neurones (Bahjaoui-Bouhaddi *et al,* 1994) suggesting the mechanisms regulating the orexin and MCH neurones are different.

Orexin receptor 2 (specific for both orexin-A and orexin-B) expression has been found in the LHA (Trivedi *et al*, 1998). Compared with aCSF, orexin-A significantly increased the number of Fos positive cells in the LHA in both virgin and pregnant groups (Fig. 5.9). Pregnant rats given orexin-A also showed a significantly greater number of Fos positive cells in the LHA than in virgin rats given orexin-A; the phenotype of cells expressing Fos in the LHA was not been determined in the present study.

Hypothalamic expression of orexin is significantly decreased during pregnancy (Garcia *et al,* 2003). However it is thought that orexin may not be directly involved in the regulation of the increased food intake observed during pregnancy (Lopez et al, 2000). Pregnancy has been associated with changes in sleep patterns. In late pregnancy there is enhanced sleep during the dark period and sleep loss during the day time (Kimura *et al,* 1996) and LHA orexin neurones have been importantly implicated during this time (Alam *et al,* 2002).

VMH. Orexin-A did not significantly increase the number of Fos positive cells in the VMH of virgin rats compared with the aCSF-treated virgin group (Fig. 5.11). In contrast, orexin-A significantly increased Fos expression in the VMH in late pregnant rats, compared with aCSF-treated pregnant rats and with both aCSF and orexin-A-treated virgin rats. The VMH has long been known to play a key role in the control of satiety. The concept that the VMH is the satiety centre in the brain whereas the LHA is the hunger centre was put forward by Elliot Stellar in 1954 (Stellar *et al,* 1954). VMH neurones are activated when food intake is stimulated in hungry rats (Johnstone *et al,* 2006). This indicates that the inhibitory action of the VMH is initiated with stimulation of feeding, although, unlike in the Johnstone *et al*, study (2006) rats in the present study were not fasted. It is notable that in the late pregnant rats Fos expression was more strongly stimulated in the VMH by orexin-A than in virgin rats, while in virgin rats orexin-A did not increase Fos expression in the VMH. This indicates activation by orexin-A of the VMH only in the pregnant rats. Orexin receptor 1, which specifically binds orexin-A, has been found in the VMH (Marcus *et al,* 2001). Nothing to date is known about orexin-receptor

expression during late pregnancy, such changes may explain increased activation of the LHA and VMH at this stage.

DMH. Orexin-A did not significantly increase Fos expression in the DMH in either the virgin or pregnant rats compared to aCSF-treated groups. Orexin receptor 1 (which is specific for orexin-A) expression has been shown in the DMH. Food deprivation has been shown to increase the expression of orexin receptor 1 as well as to increase coupling to adenylate cyclase and PLC dependent cascades (Karteris *et al,* 2005). Previous studies of responses to centrally administered orexin-A have not shown increased Fos expression in the DMH indicating that the effects of orexin-A on the HPA axis and feeding is not mediated through activation of neurons in the DMH (Harris *et al,* 2005).

5.4.3. The effect of i.c.v. orexin-A on blood glucose concentration

The results show that i.c.v. orexin-A significantly increased blood glucose concentration in virgin rats but did not significantly increase blood glucose concentration in pregnant rats (Fig. 5.17). The LHA contains glucose-sensitive neurones that are stimulated by decreased circulating glucose and inhibited by signals such as the presence of food or an increase in glucose concentration (Cai *et al,* 2001). During late pregnancy there is an impairment of insulin's ability to stimulate glucose uptake in peripheral tissues and to suppress hepatic glucose production (Leturque *et al,* 1986). This resistance is in part compensated for by insulin hypersecretion in late pregnancy (Rossi *et al,* 1993), and basal blood glucose levels decline as pregnancy progresses (Rossi *et al,* 1993). The increase in blood glucose concentration seen in virgin rats after i.c.v. orexin-A may be a biomarker of

the increase in circulating gluococorticoids that is not seen in late pregnancy, or a result of increased sympathetic drive, or increased GH, glucagon or adrenaline secretion.

The depressed basal plasma glucose level in late pregnant rats might reflect demand from the fetuses or altered hepatic sensitivity to several hormones. The finding that i.c.v. orexin-A significantly increased blood glucose in virgin rats but not pregnant rats may be due to altered hepatic sensitivity to these factors, or to failure of orexin-A to activate these systems. The HPA axis response to orexin-A is suppressed in pregnant rats (Brunton *et al,* 2003), but altered effects on other neuroendocrine systems are not known. The lower basal blood glucose concentrations in pregnant animals compared to virgins were not affected by naloxone, which had no further effect on blood glucose concentration in virgin rats. This suggests that the lack of orexin-A's effectiveness in pregnant rats is not due to a lack of corticosterone as the ACTH response was partially restored following administration of naloxone unlike the blood glucose response.

5.4.4. The effect of i.c.v. orexin-A on the HPA axis and the role of endogenous opioids

Centrally administered orexin-A significantly increased plasma ACTH concentration in virgin but not pregnant rats. This result is consistent with previous studies (Brunton *et al,* 2003). In the present study naloxone had no further effect on plasma ACTH secretion in virgin rats but restored an ACTH response in pregnant rats (Fig. 5.18).

A common central opioid mechanism has been shown to inhibit neuroendocrine stress responses during late pregnancy (Douglas *et al,* 1998). Naloxone is likely to be exerting its effects on the HPA axis via the hypothalamus (Wang *et al,* 1996) and thus may affect hypothalamic pituitary mechanisms through CRH release. μ-opioids appear to mediate the naloxone-induced reduction in stress responses (Cover & Buckingham, 1989), whereas κ- and δ-opioids modulate HPA axis hormone secretion under basal conditions (Iyengar *et al,* 1986). Hyporesponsiveness of the HPA axis to immune challenge during late pregnancy is a result of endogenous opioid inhibition of the pPVN CRH neurones (Brunton *et al,* 2005). This endogenous opioid inhibition may be exerted by the enkephalins produced by NTS neurones and acting via μ-opioid receptors on noradrenergic terminals in the PVN. Proenkephalin mRNA expression is increased during late pregnancy in the NTS (Brunton *et al,* 2005). Orexin neurones project widely throughout the brain including projections to the NTS (de Lecea *et al,* 1998). It has been shown following administration of IL-1β that in late pregnancy opioids inhibit CRH neurone responses by acting presynaptically on noradrenergic inputs to the CRH neurones (Brunton *et al,* 2005); it was proposed for the present study that opioids might similarly interfere with orexin signalling to the pPVN CRH/AVP neurones.

Orexin-A significantly increased CRH and AVP mRNA expression in the pPVN in virgin rats, but had no such effect in late pregnant rats, confirming previous findings (Brunton *et al*, 2003). In the present study, naloxone restored pPVN AVP and CRH mRNA responses to orexin-A in pregnant rats, indicating opioid inhibition of orexin signalling to the CRH/AVP pPVN neurones in late pregnancy. Naloxone had no effect in virgin rats, supporting previous studies that showed activation of an opioid

mechanism inhibiting HPA axis responses in pregnancy (Brunton *et al*, 2005). The action of naloxone in late pregnant rats might be a consequence of inhibition of NPY signalling to the HPA axis, or interference with orexin-A acting via the NTS, or via direct signalling to the HPA axis in late pregnancy.

The HPA axis response to central administration of NPY has also been shown to be suppressed during late pregnancy (Brunton *et al,* 2006). The aim of the next experiments was to investigate if the endogenous opioid inhibition seen in response to orexin-A was interfering with NPY signalling to the PVN CRH/AVP neurones. NPY has also been shown to stimulate oxytocin secretion which action is suppressed during late pregnancy (Brunton *et al,* 2006). Endogenous opioids have been shown to inhibit oxytocin neurones during late pregnancy also (Douglas *et al,* 1998).

5.4.5. Summary

Feeding and Fos responses to orexin A during late pregnancy

Orexin-A stimulated eating behaviour in both virgin and pregnant rats. Naloxone did not affect eating response to orexin-A in either the virgin or the pregnant group, indicating orexin-A stimulates eating behaviour independently of opioids. Orexin-A did not significantly stimulate drinking in either the virgin or the pregnant group yet it increased grooming in the virgin rats with a tendency for grooming to increase in the pregnant rats. I.c.v. orexin-A increased the number of Fos-positive cells in the pPVN of virgin rats compared to virgin controls and compared to pregnant rats given orexin A, indicating reduced responsiveness of these neurones during late pregnancy. NPY neurones in the ARC have been shown to play a major role in regulating appetite. There was a significantly greater number of Fos positive cells in the ARC in virgin rats given orexin compared to pregnant rats. Thus it seems the lack of

activation of ARC NPY neurones by orexin-A during late pregnancy is not interfering with the actions of orexin-A in stimulating eating behaviour. Orexin-stimulated feeding in late pregnancy may be activated by direct orexin-A actions on the LHA. The increased Fos expression in the VMH in pregnant rats given orexin-A suggests that the inhibitory action of the VMH is initiated with stimulation of feeding although nothing to date is known about orexin receptor expression during late pregnancy. Altered orexin receptor expression may explain increased activation of the VMH and LHA during late pregnancy. Orexin-A did not increase Fos expression in the DMH.

HPA axis responses to orexin-A during late pregnancy

Centrally administered orexin-A increased plasma ACTH concentration in virgin but not late pregnant rats. Naloxone had no further effect on plasma ACTH secretion in virgin rats but restored an ACTH response in pregnant rats. Orexin-A significantly increased CRH and AVP mRNA expression in the PVN in virgin rats but had no such effect in pregnant rats. Naloxone again restored pPVN AVP and CRH mRNA responses to orexin-A in pregnant rats.

Reduced pPVN Fos responses indicate reduced responsiveness of the CRH/AVP neurones in pregnancy. It seems that endogenous opioids are interfering with orexin-induced signalling to the HPA axis during late pregnancy.

Opioid inhibition of the HPA axis responses at the level of the CRH/AVP neurones may account for reduced orexin-A actions in pregnancy. Orexin-A acts on the HPA axis in part via the ARC NPY neurones, so the aim of the next experiments was to investigate if the endogenous opioid inhibition seen on responses to orexin-A was interfering with NPY signalling to the PVN CRH/AVP neurones.

NPY

6.1 Neuropeptide Y (NPY)

NPY is a 36 amino acid polypeptide that belongs to the pancreatic family of polypeptides. High levels of NPY are detected in the central and peripheral nervous system of humans and other animals (Heilig *et al,* 1995).

The distribution of central NPY-pathways is summarised in Figure 6.1.

NPY can function as a neurotransmitter or a neuromodulator. Its action is mediated by specific receptors, Y1, Y2, Y3, Y4, Y5 and Y6 (Balasubramaniam *et al,* 1997).

There are several major effects of NPY on the central nervous system which include stimulating food intake, an anti-anxiety effect, controlling circadian rhythms and regulating hormone synthesis (White *et al,* 1993).

6.1.1. Distribution of NPY

In the central nervous system NPY is prominently synthesised in the arcuate nucleus (ARC). It is also synthesised in the brain stem in the nucleus of the solitary tract (NTS) (Heilig *et al,* 1995). In the brain stem NPY is colocalised with adrenaline and noradrenaline (Heilig *et al,* 1995). NPY neurones project to many areas in the brain, particularly to the paraventricular nucleus (PVN) as well as to the supraoptic nucleus (SON), the ARC, the dorsal medial hypothalamus (DMH) and the ventro-medial hypothalamus (VMH) (Li *et al,* 1994). NPY neurones also project to the medial preoptic area, the lateral hypothalamic area (LHA) and the NTS (de Quidt *et al,* 1986).

NPY is considered to be the most abundant and widely distributed neuropeptide present in the mammalian central and peripheral nervous system. In the periphery it is generally found in the sympathetic nervous system; co-stored and co-released with

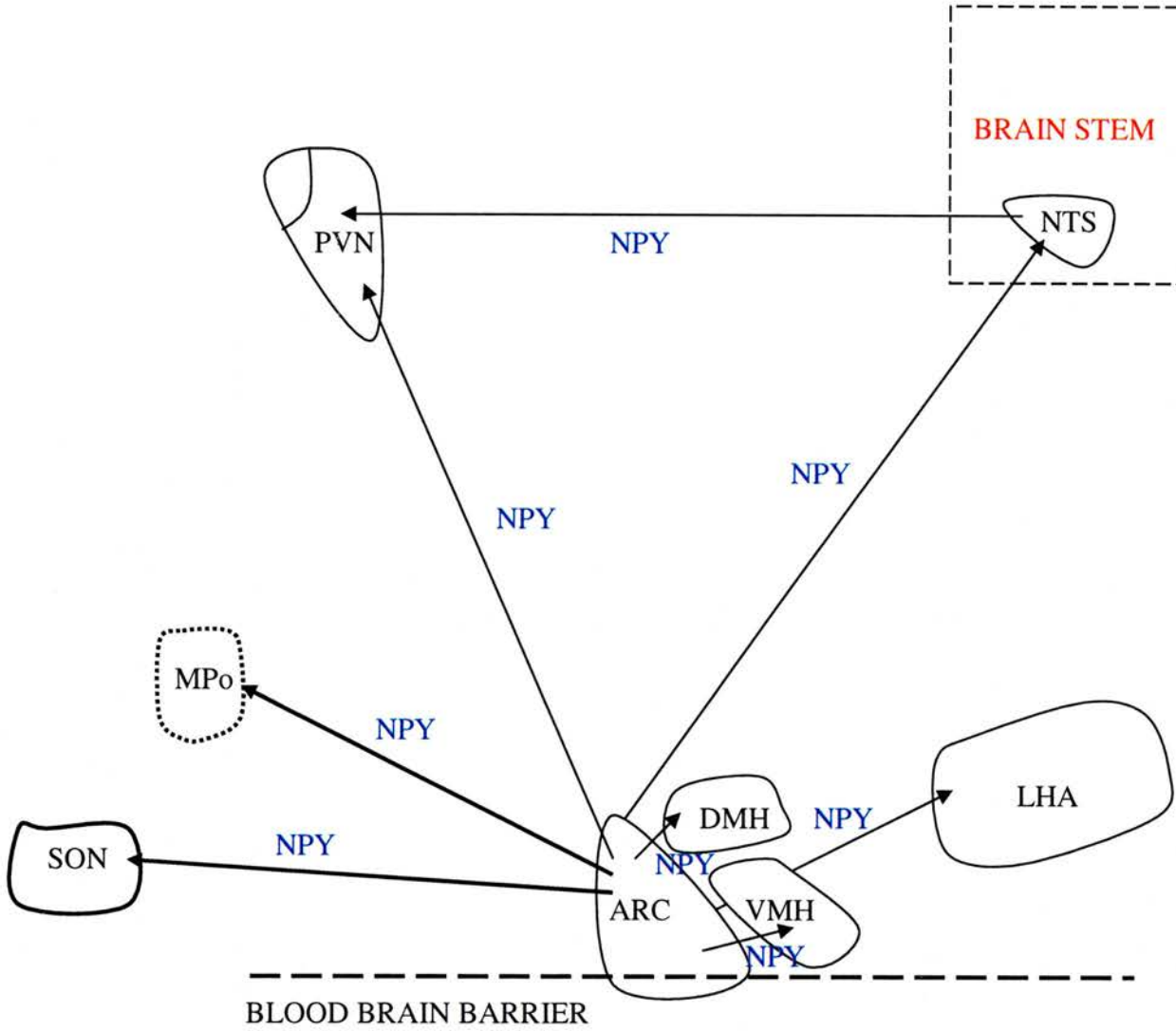


Figure 6.1: Centrally projecting NPY pathways. ARC = arcuate nucleus, VMH = ventromedial hypothalamus, DMH = dorsomedial hypothalamus, LHA = lateral hypothalamic area, PVN = paraventricular nucleus, MPo = medial preoptic area, SON = supraoptic nucleus, NTS = nucleus of the solitary tract

noradrenaline (Heilig *et al,* 1990). NPY has also been found in several other organs including the GI tract, salivary glands, thyroid gland, pancreas, urogenital system, the airways and the heart (Jackerott *et al,* 1997).

The distribution of NPY in the central nervous system is complex. In general it is distributed in similar neurones (with somatostatin and GABA) in the cerebral cortex and forebrain, whilst it is also found in a variety of other neurones in the brain containing either noradrenaline, adrenaline or serotonin in other areas of the brain (Heilig *et al,* 1990). NPY is found in high concentrations in the hypothalamus, and periventricular areas contain the highest density of NPY while the lowest quantities are found in the mammillary bodies (Kalra *et al,* 1991).

6.1.2. The NPY receptors

All the NPY receptors cloned to date possess seven transmembrane domains characteristic of the G-protein coupled super-family of receptors.

Y1 receptor

There is high expression of the Y1 receptor in the colon, kidney and placenta (Wharton *et al,* 1993). Y1 receptors have also been found in high quantities in ganglia, the myenteric and submucous plexuses, submucosal blood vessels and the fetal adult myocardium (Wharton *et al,* 1993). Y1 receptors are coupled to both cyclic AMP (cAMP) (Fuhlendorff *et al,* 1990) and also calcium channels (Daniels *et al,* 1992). It is thought that the action of feeding on NPY is mediated through the Y1 receptor (McLaughlin *et al,* 1991).

Y2 receptor

The Y2 receptor possesses a 30% homology with the Y1 receptor (Rose *et al,* 1995). Y2 receptors are expressed in chromaffin cells (Gerald *et al,* 1995) and have been found in nerve endings, hippocampus, renal proximal tubular cells, the hypothalamus and dorsal root ganglia (Aicher *et al,* 1991). They are also coupled to intracellular calcium stores as well as cAMP (Bleakman *et al,* 1991).

Y3 receptor

It has been suggested that several types of Y3 receptor exist (Balasubramaniam *et al,* 1990). Y3 receptors have been identified in chromaffin cells (Wahlestedt *et al,* 1992), cardiac ventricular cells (Balasubramaniam *et al,* 1990) and also in the brainstem (Grundemar *et al,* 1994). The Y3 receptors have all been shown to be coupled to adenylate cyclase (Harfstrand *et al,* 1987).

Y4 receptor

The human (Bard *et al,* 1995), rat (Lundell *et al,* 1996) and mouse (Gregor *et al,* 1996) pancreatic polypeptide (PP) receptor was cloned and defined as the Y4 receptor. The human form only shares 75% homology with the rat form. Y4 receptors are located mainly in the human colon, but are found in the testis, lungs and heart in the rat and mouse. Y4 receptors are also linked to cAMP and to calcium stores (Bard *et al,* 1995).

Y5 receptor

It is suggested that the Y5 receptor may well also be involved in NPY-induced feeding (Gerald *et al,* 1996). Y5 receptors have been found in the dentate gyrus, hippocampus and in parts of the hypothalamus.

Y6 receptor

The Y6 receptor has been identified as another form of the Y5 receptor (Hu *et al,* 1996). This is found to be highly localised in the hypothalamus.

6.1.3. The function of NPY in the central nervous system

A large number of studies have focussed on the potential roles played by NPY within the hypothalamus and pituitary with respect to the control of food intake and energy homeostasis (White *et al,* 1993). NPY is one of the most powerful orexigenic agents known. When administered icv in rats it produces a powerful and prolonged increase in food intake (Clark *et al,* 1984). When administered chronically it produces hyperphagia, decreased thermogenesis and obesity (Stanley *et al,* 1986). The Y1, Y2 and Y5 receptors are found in the hypothalamus. Y1 and Y5 receptors have been implicated as important receptors that mediate the feeding effects of NPY (Marsh *et al,* 1998).

NPY has also been shown to be involved as an anti-anxiety agent. Many studies have found reduced concentrations of NPY in cerebrospinal fluid (CSF) or plasma from depressed patients (Mathé *et al*, 1996). In many models of depression rats have been found to have reduced levels of NPY in the hippocampus and exaggerated mobility (Holmes *et al,* 1998). Central administration of NPY reduces immobility to a similar level as imipramine (Husum *et al,* 2000). Taken together these studies suggest an antidepressant-like profile of NPY and support the hypothesis that NPY plays a role in the pathophysiology of depression.

NPY also plays a role in modulating mammalian circadian rhythms (Soscia *et al,* 2004). NPY resets the circadian clock during the subjective day, mediating non-

photic inputs (Hannibal *et al,* 2002). Previous studies using receptor-selective agonists have indicated that this action of NPY is mediated by the Y2 receptor in hamsters (Golombek *et al,* 1996).

Finally NPY also plays a role in regulating hormone synthesis. NPY influences the synthesis of luteinising hormone (Wankowska *et al,* 2002) thyrotrophin releasing hormone (TRH) and melanocyte concentrating hormone (MCH) (Korner *et al,* 2003). NPY also regulates the growth hormone (Peng *et al,* 1993) and glucococorticoid axes (Hanson *et al,* 1995). It is widely believed then that hypothalamic and pituitary NPY-expressing cells represent an important and critical site of integration of peripheral hormonal signals with regulation of energy homeostasis (White *et al,* 1993).

6.1.4 The role of NPY in regulating the HPA axis

The PVN is the main site where nerve terminals of NPY-containing neurones are found. The PVN, especially the parvocellular area, is the main site of synthesis of corticotrophin releasing hormone (CRH) in the hypothalamus (Liposits *et al,* 1988). Electron microscopy has shown that there are synapses between neurones containing NPY and the cell bodies of CRH neurones (Liposits *et al,* 1988). The synapses are asymmetrical in shape indicating a stimulatory transmission of activity (Cohen *et al,* 1982). *In vivo* (Haas *et al,* 1987) and *in vitro* (Tsagarakis *et al,* 1993) studies have shown that NPY stimulates CRH release. After i.c.v. administration of NPY the level of POMC mRNA in the anterior pituitary also increases (Suda *et al,* 1993) as does the release of adrenocorticotropic (ACTH) hormone (Haas *et al,* 1989). NPY inhibits GABA neurones around the PVN (Fu *et al,* 2004). So NPY action in the pPVN causes overall activation of the HPA axis. *In vivo* the HPA axis can be stimulated by

NPY given via a variety of routes, either intra-venously, through the lateral ventricle (Suda *et al,* 1993) into the third ventricle (Brooks *et al,* 1994) or directly into the PVN (Haas *et al,* 1989). The greatest CRH release occurred when NPY was injected directly into the PVN suggesting its importance in the activation of the HPA axis by NPY. The permeability of the blood-brain barrier to NPY is low so intravenous administration does prove to be less effective.

It seems that NPY directly affects CRH release (Suda *et al,* 1993), but an indirect effect through adrenergic receptors cannot be excluded (Haas *et al,* 1989). The NPY receptor that mediates an increase in HPA axis activity is as yet still unknown.

It is also likely that the pituitary gland is involved in regulating the HPA axis response to NPY because large amounts of NPY are released from the hypothalamus into the pituitary portal circulation (McDonald *et al,* 1987). It is thought that NPY acts on the pituitary to stimulate GH release (Peng et al, 1990) and possibly ACTH release (Ruiz-Gayo *et al*, 2000).

NPY is also likely to be involved in direct regulation of activity of the adrenal glands. NPY-containing nerve terminals have been found in the zona glomerulosa (Mazzocchi *et al,* 1987) though it seems that the role of NPY in the physiological regulation of the HPA axis is of less importance at the level of the adrenal glands than in the hypothalamus.

6.1.5 The effect of NPY on the HPA axis in pregnancy

It has previously been shown that during late pregnancy the excitatory effects of orexin-A on HPA activity are suppressed as a result of reduced activation of CRH neurones in the pPVN. NPY stimulates CRH release from the median eminence

(Haas *et al,* 1987), increases CRH mRNA expression in the pPVN and increases plasma concentrations of ACTH (Haas *et al,* 1989) and corticosterone (Harfstrand *et al,* 1987). Orexin-A stimulates NPY release from hypothalamic explants (Lang *et al,* 1983), so its actions on the HPA axis may be mediated by NPY neurones.

During late pregnancy secretion of oxytocin, a stress hormone, in response to emotional and physical stressors is reduced (Neumann *et al,* 1998). Although neuroendocrine stress responses to centrally administered NPY are absent in late pregnant rats, behavioural responses are intact (Brunton *et al,* 2006); absent HPA axis responses to NPY are in pregnancy due to decreased activation of the CRH/AVP pPVN neurones. A lack of oxytocin secretory responses to NPY in late pregnancy is a result of a failure to activate SON and mPVN oxytocin neurones (Brunton *et al,* 2006).

6.1.6 Aims of these experiments

Reduced HPA axis responses to centrally administered orexin-A in pregnancy are evidently a result of a reduced response of the CRH neurones to NPY at this time. Endogenous opioids act to inhibit supraoptic oxytocin neurones in late pregnancy (Douglas *et al,* 1995); it is not known whether in late pregnancy opioids interfere with NPY signalling to pPVN CRH and/or AVP neurones, or to supraoptic oxytocin neurones. NTS neurones may be the source of the endogenous opioid, since proenkephalin mRNA expression is up regulated in the NTS during late pregnancy. Opioids inhibit CRH neurone responses following cytokine challenge during late pregnancy by acting presynaptically on noradrenergic inputs to the CRH neurones (Brunton *et al,* 2005). In chapter 5 we showed endogenous opioids interfere with

orexin signalling to the HPA axis in pregnancy. Here we investigated activation of hypothalamic neurones regulating HPA responses to NPY, and whether opioids were interfering with NPY signalling to pPVN CRH/AVP or SON oxytocin neurones. The two main aims of this experiment were to investigate hypothalamic circuitry regulating HPA and eating responses to NPY during pregnancy and to investigate the role of endogenous opioids to see if, like their actions on orexin-A, opioids are interfering with NPY signalling to the HPA axis during late pregnancy.

6.2 Methods

6.2.1. Animals

Female Sprague Dawley rats were used and housed individually after surgery. Rats were maintained as described in section 2.1.

6.2.2. Surgery

Rats were implanted with an intracerebroventricular cannula for experiment one and an intracerebroventricular and jugular vein cannula in experiments two and three. Surgery was performed under conditions described in section 2.3.

6.2.3 Experiment 1 – The effect of centrally administered NPY in pregnancy

On the day of the experiment (day 21 of pregnancy), rats were left undisturbed for 90 minutes. At between 07:30-08:30 rats were given either NPY (1nmol in 2µl) or vehicle (artificial cerebro-spinal fluid, aCSF; pH 7.2, composition in mM: NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; NaH_2PO_4, 0.49; $CaCl_2$, 1.26; $MgCl_2$, 1.18) which was given i.c.v. with gentle restraint over a period of about 30

seconds. The following behaviours were noted continually: inactive, grooming, oral motor activity, eating and drinking. Behavioural data were collected for 90 minutes; an event was classified as occurring if an animal spent $\geq$ 5 seconds exhibiting the behaviour. Rats were killed by transcardial perfusion-fixation under deep anaesthesia 90 minutes after NPY (this time point has previously been shown as the optimum time for showing increased Fos expression in the central nervous system after NPY) (Niimi *et al,* 2001). Rats were examined post-mortem to check fetuses and i.c.v. cannula placement. Brains were processed for Fos immunohistochemistry on free floating sections and Fos expression measured.

6.2.4 Experiment 2 – The effect of endogenous opioids on reduced HPA responses to NPY in pregnancy

On the day of the experiment (day 21 of pregnancy) the cannulae were connected between 07:30-08:30h. The jugular vein cannula was attached to PVC extension tubing led out of the cage and connected to a 1ml syringe filled with heparinised saline (0.9% saline, 50 units/ml). Rats were left undisturbed for 90 minutes and a basal blood sample (0.55ml) was taken. Immediately after the first basal blood sample either naloxone (10mg/ml) or saline both at 0.5ml/kg were given i.v.; 15 minutes after the first basal blood sample another basal blood sample was taken, and a further 15 minutes later rats were given either NPY (1nmol in 2μl) or vehicle (artificial cerebro-spinal fluid, aCSF; pH 7.2, composition in mM: NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; NaH_2PO_4, 0.49; $CaCl_2$, 1.26; $MgCl_2$, 1.18) i.c.v. with gentle restraint over a period of about 30 seconds. Further blood samples (0.55ml) were taken for glucose measurement and for ACTH assay at

15, 30, 60 and 120 minutes after i.c.v. infusion. Blood samples were placed into eppendorfs containing 50µl of chilled 5% EDTA. After each blood sample, blood was replaced with 0.9% sterile saline. Glucose measurements were taken throughout the experiment using a Roche Accuchek Active Meter. Plasma was separated by centrifugation and stored at -20°C until radioimmunoassay. Rats were killed by conscious decapitation 4 hours after NPY (this time point has previously been shown as the optimum time for showing increased CRH mRNA expression in the PVN following stress) (Harbuz *et al,* 1989). Rats were examined post-mortem to check fetuses and i.c.v. cannula placement. Blood samples were processed for ACTH assay and brain sections processed for CRH and AVP *in situ* hybridisation.

6.2.5 Experiment 3 – Fos expression in response to NPY after administration of naloxone

On the day of the experiment (day 21 of pregnancy) the jugular vein cannula was attached to PVC extension tubing led out of the cage and connected to a 1ml syringe filled with heparinised saline (0.9% saline, 50 units/ml). Rats were left undisturbed for 90 minutes and then given either naloxone (10mg/ml) or saline, both at 0.5ml/kg i.v.; 30 minutes later rats were given either NPY (1nmol in 2µl) or vehicle (artificial cerebro-spinal fluid, aCSF; pH 7.2, composition in mM: NaCl, 138; KCl, 3.36; $NaHCO_3$, 9.52; Na_2HPO_4, 0.49; urea, 2.16; NaH_2PO_4, 0.49; $CaCl_2$, 1.26; $MgCl_2$, 1.18) i.c.v. with gentle restraint over a period of about 30 seconds. The following behaviours were noted continually: inactive, grooming, oral motor activity, eating and drinking. Behavioural data were collected for 90 minutes; an event was classified as occurring if an animal spent $\geq$ 5 seconds exhibiting the behaviour. Pre-

weighed food was given to the rats and re-weighed after the experiment. Rats were killed by transcardial perfusion-fixation under deep anaesthesia 90 minutes after NPY (this time point has previously been shown as the optimum time for showing increased Fos expression in various brain areas following administration of a stressor (Niimi *et al,* 2001). Rats were examined post-mortem to check fetuses and to check i.c.v. cannula placement. Brains were processed for Fos immunohistochemistry on free floating sections and Fos expression measured.

6.2.6. Fos immunohistochemistry

Brain sections (52μm) were cut through the PVN, SON, ARC, LHA, VMH and DMH on a freezing microtome onto gelatinised slides. Fos immunohistochemistry was performed using the method described in section 2.5.2. with free floating sections. Sections for double labeling were further washed in PB-T for 2 x 5 min and incubated in 0.3% hydrogen peroxide for 15 minutes, washed again in PB-T before being incubated in an anti-rabbit oxytocin antibody (dilution 1:1000 in PB-T containing 1% NSS; Calbiochem, Nottingham, UK) for 24 hours. Sections were then washed and incubated with the ABC kit described in section 2.5.2. Sections were visualised by incubating sections in 0.1M PB containing 0.025% DAB and 0.03% hydrogen peroxide. The reaction was stopped in 0.1M PB. Fos-positive stained cells were counted in each region with at least 3 sections counted per rat.

6.2.7 In Situ Hybridisation

Brains were cryostat sectioned at 15μm and mounted onto Polysine slides (BDH; see section 2.4.2). To detect CRH mRNA expression a 42-mer oligonucleotide probe

(MWG-Biotech) was used complementary to bases 496-537, which encode amino acids 22-35 of the rat CRH peptide (Jingami *et al*, 1985). The probe sequence used was:

5'-CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GCC- 3'

To detect AVP mRNA expression a 36-mer oligonucleotide probe (MWG-Biotech) was used, complementary to bases 486-521 of rat AVP mRNA (Majzoub *et al,* 1983). The probe sequence used was:

5'-GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT-3'

Both probes were labeled with ^{35}S as described in section 2.4.2. The melting temperature for the CRH mRNA probe was 78°C and for the AVP mRNA probe was 76°C, therefore the heated SSC washes were performed at 58°C and 56°C respectively. Once dry the sections were dipped in photographic emulsion under safelight conditions and stored at 4°C. The exposure time was 13 weeks for CRH mRNA and 7 days for AVP mRNA. The number of positive cells and the grain density were measured by the image analysis system described in 2.4.2.

6.2.8 ACTH radioimmunoassay

Plasma ACTH concentration was determined using a commercially available kit as described in section 2.7.3. The sensitivity of the ACTH assay was 11pg/ml. The intra-assay variation was <7%.

6.2.9 Statistics

A two-way analysis of variance (ANOVA) was used with a repeated measures ANOVA for blood sampling experiments. A significant ANOVA ($p<0.05$) was

followed by Student Newman Keuls multiple comparison tests, to identify differences. A p value of less than 0.05 was considered statistically significant.

6.3 Results

6.3.1 Experiment 1 – The effect of centrally administered NPY in pregnancy

Behavioural data

Analysis of behavioural data using a two-way ANOVA showed a statistically significant difference in eating behaviour following i.c.v. NPY administration ($p<0.001$). NPY significantly increased eating behaviour in virgin and pregnant rats ($p<0.001$; two-way ANOVA) (Fig.6.2). There were no significant differences in drinking between groups (Fig.6.2). There was a statistically significant difference in grooming behaviour following NPY administration ($p=0.017$) (Fig.6.2). NPY significantly increased grooming behaviour in virgin rats ($p=0.019$; two-way ANOVA) (Fig.6.2).

Parvocellular paraventricular nucleus (pPVN) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p=0.010$). Quantification of Fos positive cells revealed that Fos expression in the pPVN of virgin rats was significantly greater (2.5 times) than in the pregnant NPY group (Fig.6.4). I.c.v. injection of NPY significantly increased Fos expression in the virgin group ($p<0.001$) (28.1 ± 4.3 vs control 11.9 ± 1.3 Fos positive cells/PVN), but had no effect in late pregnant rats (Fig.6.3 and Fig.6.5).

Magnocellular paraventricular nucleus (mPVN) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p=0.011$). Quantification of Fos positive cells revealed that Fos expression in the mPVN of virgin rats was significantly greater (2-fold) than in the pregnant NPY

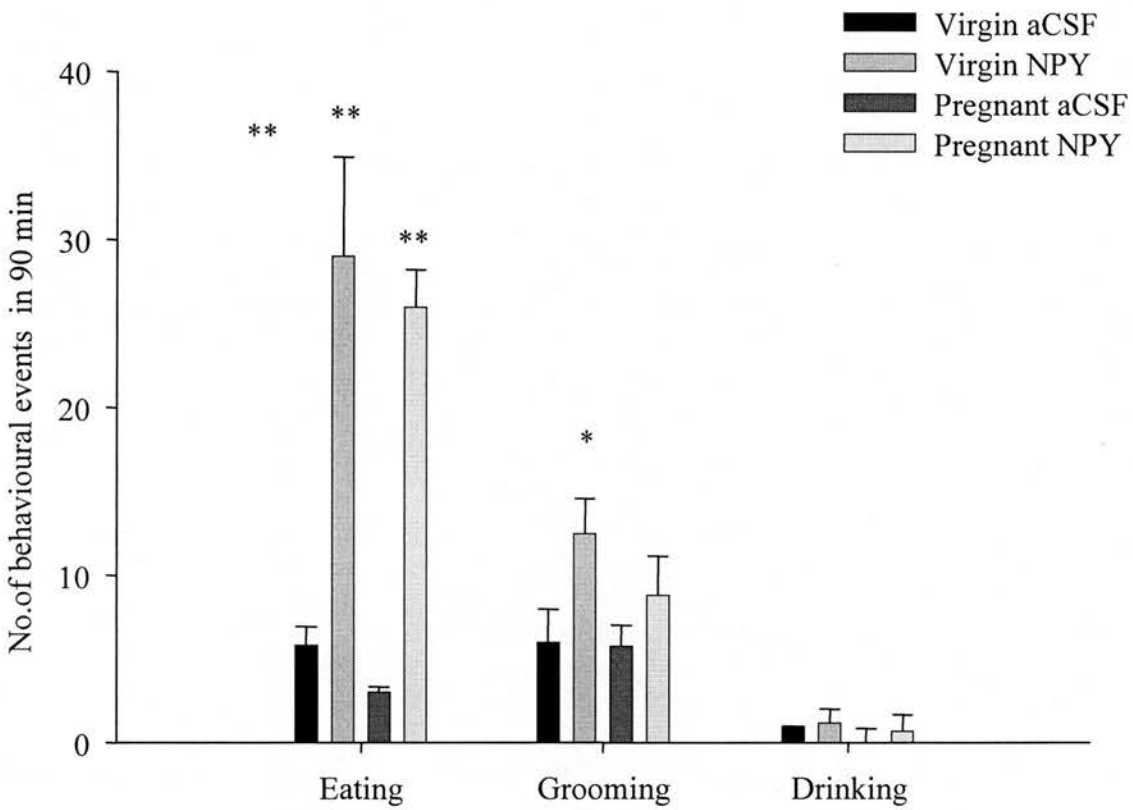


Figure 6.2: The effect of i.c.v. NPY on behaviours in virgin and pregnant rats. Values are group means ± SEM. Virgin/aCSF, n=6; virgin/NPY, n=6; pregnant/ aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: **p<0.001, *p<0.05 vs aCSF.

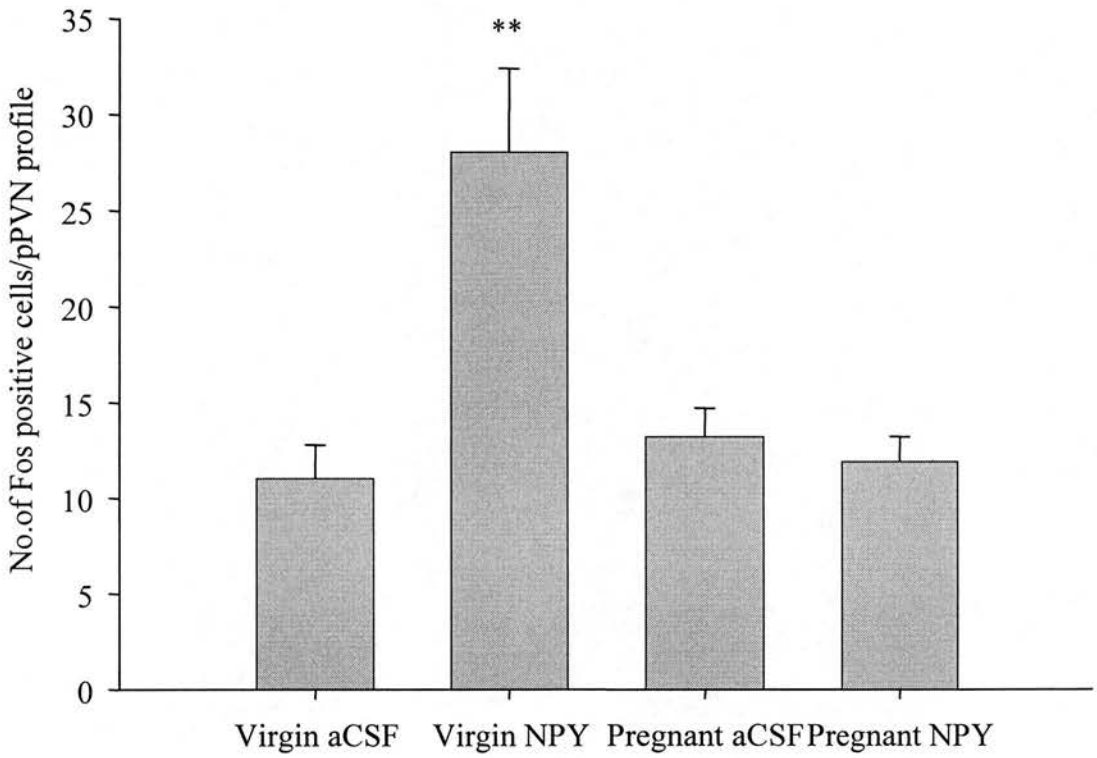


Figure 6.3: The effect of i.c.v. NPY on Fos positive cell counts in the pPVN in virgin and pregnant rats. Rats were killed by transcardial perfusion-fixation 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=6; virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test:
**$p<0.001$ vs all other groups.

group (Fig.6.4). I.c.v. injection of NPY significantly increased Fos expression in the virgin group ($p=0.004$) (15.6 ± 3.6 vs control 5.6 ± 0.8 Fos positive cells/PVN), with no significant effect in the pregnant group (Fig 6.4 and Fig. 6.5).

Analysis of Fos and oxytocin expression using a two-way ANOVA showed there was a statistically significant difference in the percentage of oxytocin neurons expressing Fos in virgin rats compared to pregnant rats ($p< 0.02$). Quantification of percentage of OT cells expressing Fos revealed that the percentage of oxytocin cells activated in the mPVN of virgin rats was significantly greater (12-fold) than in the pregnant NPY group (Fig 6.6). There were approximately 50 OT neurons counted per mPVN section. I.c.v. injection of NPY significantly increased the percentage of oxytocin neurones activated ($p < 0.001$) (12.6 ± 2.7 vs control 0.8 ± 0.5 Fos positive cells/PVN) in virgin rats, with no effect in pregnant rats (Fig 6.6). A representative photomicrograph of a Fos-positive oxytocin cell is seen in figure 6.7.

Supraoptic Nucleus (SON) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p= 0.013$). (Fig.6.8). Quantification of Fos positive cells revealed that Fos expression in the SON of virgin rats given NPY was significantly greater (2-fold) than in the pregnant NPY group (Fig.6.8). I.c.v. injection of NPY significantly increased Fos expression in the virgin group ($p<0.001$) (19.2 ± 4.4 vs control 3.9 ± 0.8 Fos positive cells/SON), with no effect in the pregnant group (Fig 6.8 and Fig 6.9). Analysis of Fos and oxytocin expression using a two-way ANOVA showed there was a statistically significant difference in the percentage of oxytocin neurones expressing Fos in virgin rats compared to pregnant rats ($p< 0.001$). Quantification of

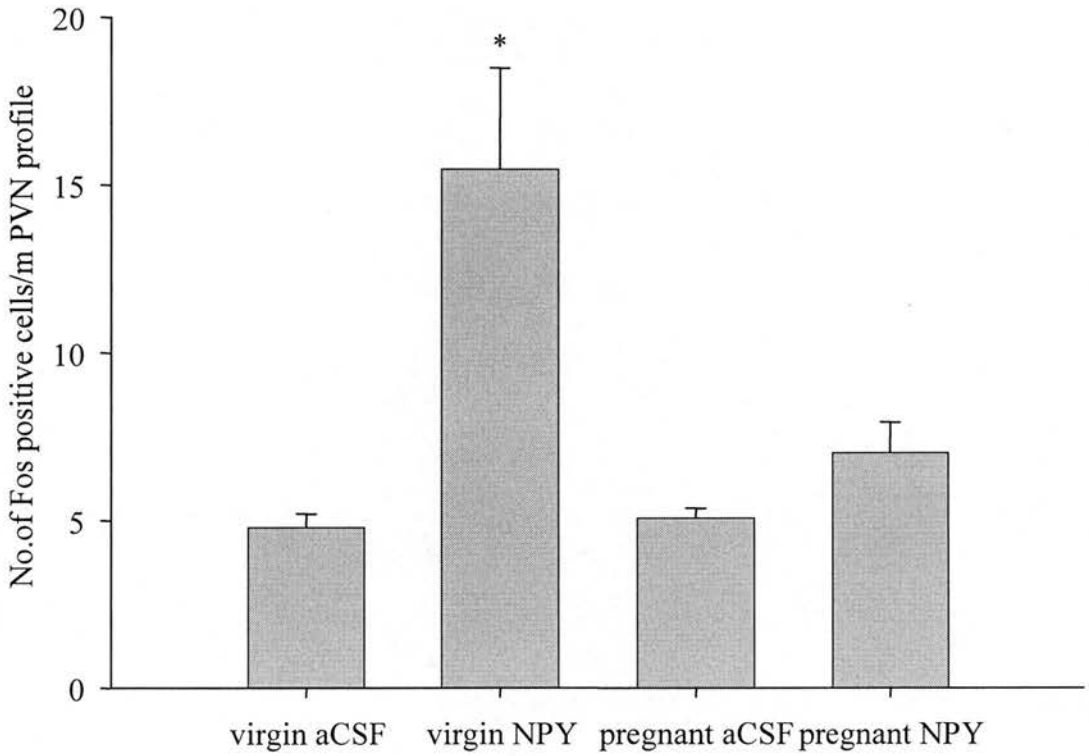


Figure 6.4: The effect of i.c.v. NPY on Fos positive cell counts in the mPVN in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n= 6; Virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs all other groups,

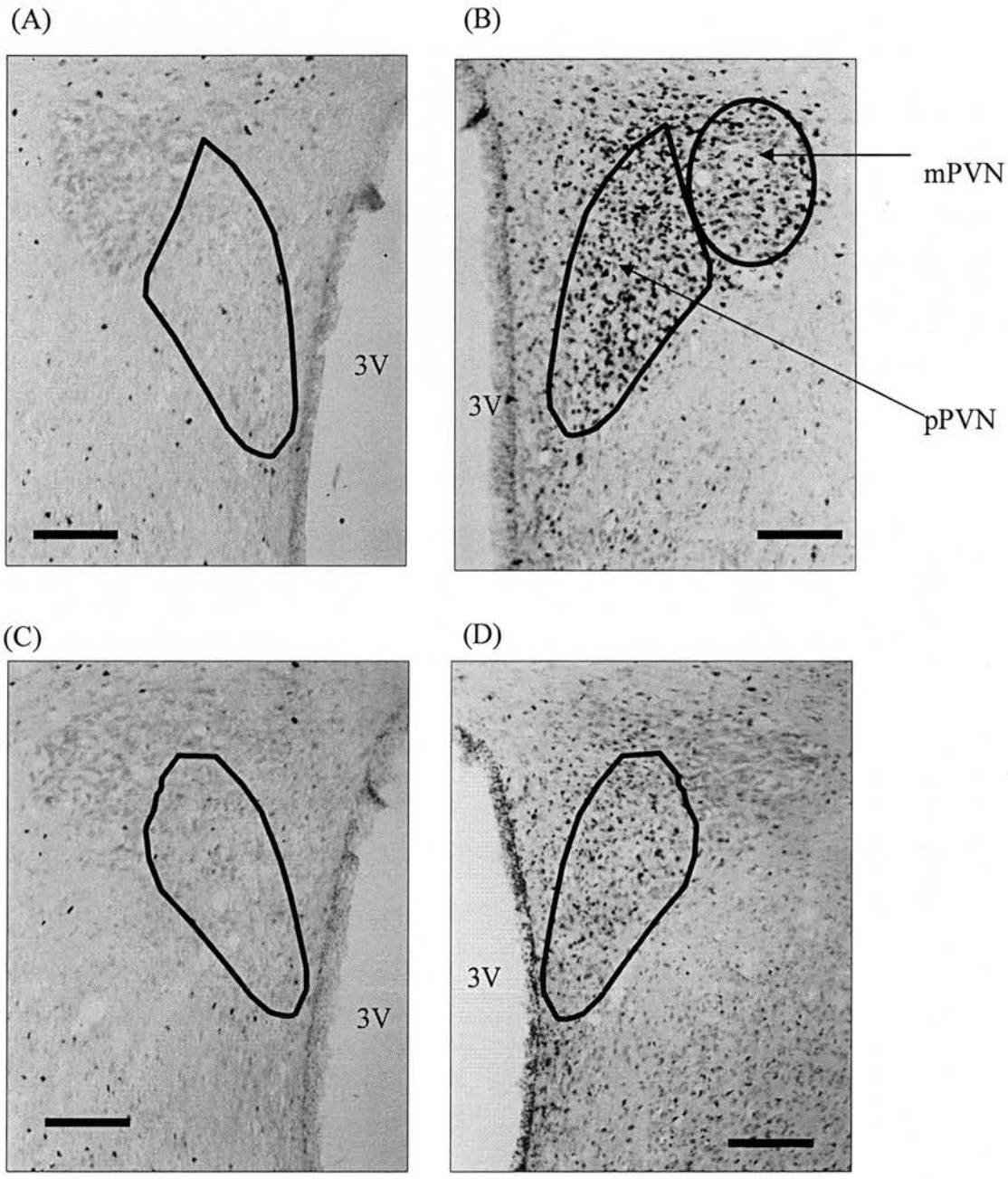


Figure 6.5: Representative photomicrographs of coronal sections through the PVN in virgin and pregnant rats. Sections were processed for Fos Immunohistochemistry: (A) virgin aCSF; (B) virgin NPY; (C) pregnant aCSF; (D) pregnant NPY. Scale bar = 100μm. 3V = 3rd ventricle mPVN = magnocellular PVN; pPVN = parvocellular PVN.

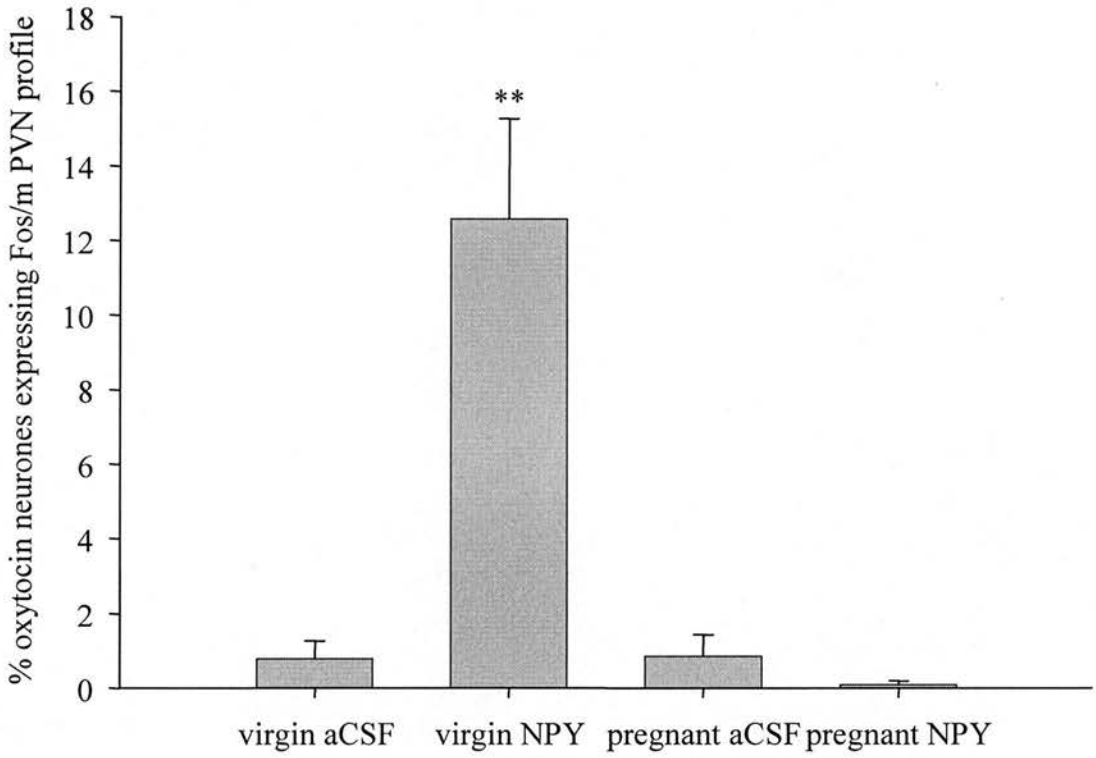


Figure 6.6: The effect of i.c.v. NPY on oxytocin neurone activation in the mPVN in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 Minutes post-injection o NPY. The number of oxytocin cells were counted and the number of oxytocin cells expressing Fos was divided by the total number and expressed as a percentage. Values are the group means ± SEM. Virgin/aCSF, n=6; Virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA Followed by a Student Newman Keuls multiple comparison test: **$p<0.001$ vs all other groups.

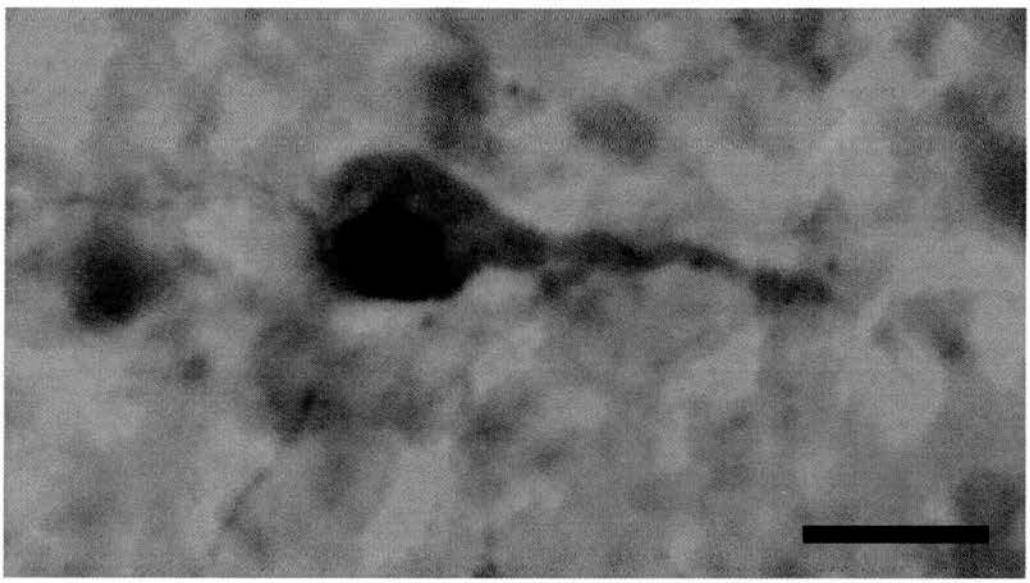


Figure 6.7 Representative photomicrograph of a double-labelled oxytocin and Fos positive cell from a mPVN section. Scale bar = 25μm.

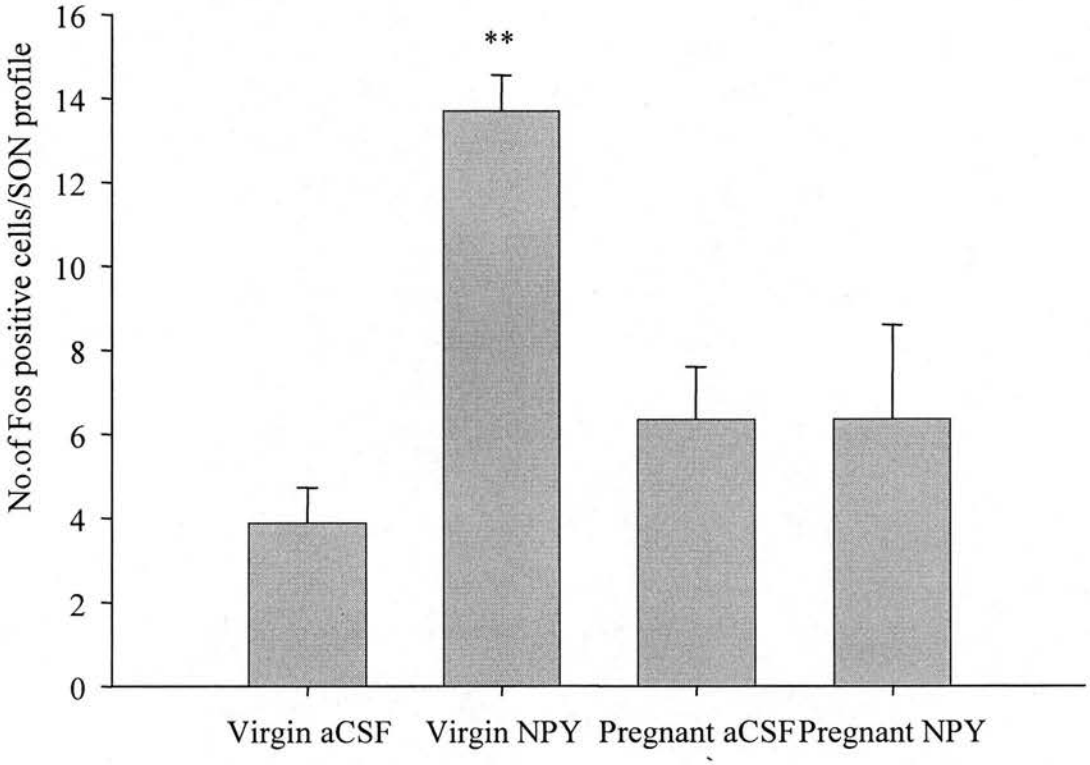


Figure 6.8: The effect of i.c.v. NPY on Fos positive cell counts in the SON in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 Minutes post-injection of NPY. Values are the mean count positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=6; Virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: ** $p<0.001$ vs all other groups.

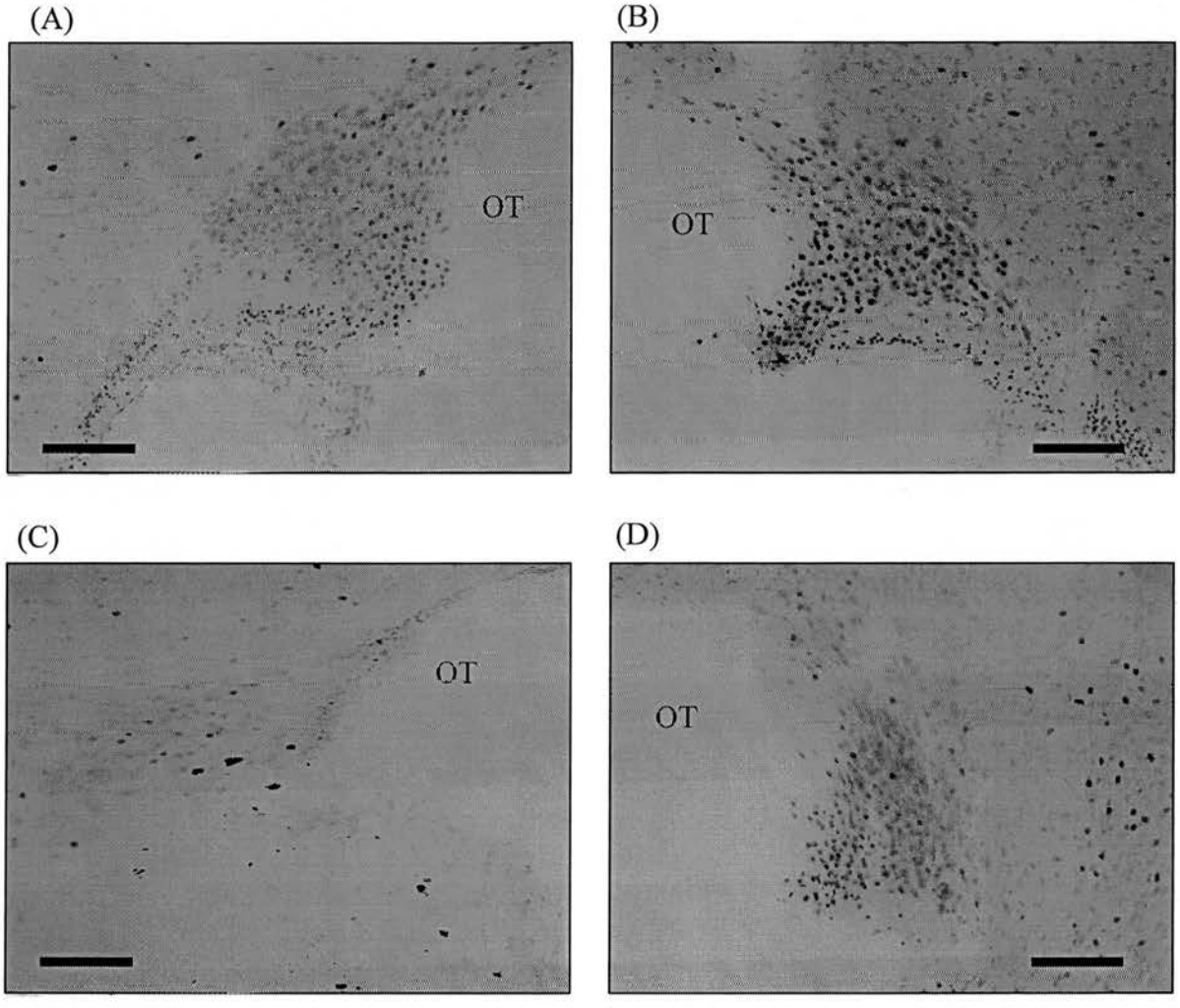


Figure 6.9: Representative photomicrographs of coronal sections through the SON in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry: (A) virgin aCSF; (B) virgin NPY; (C) pregnant aCSF; (D) pregnant NPY. Scale Bar = 100μm. OT = optic tract.

percentage OT cells revealed that the percentage of oxytocin cells activated in the mPVN of virgin rats was significantly greater (12 fold) than in the pregnant NPY group (Fig 6.10). I.c.v. injection of NPY significantly increased the percentage of oxytocin neurones activated in virgin rats ($p < 0.001$) (59.6 ± 5.3 vs control 9.5 ± 5.1 Fos positive cells/SON), with no effect in the pregnant rats (Fig 6.10).

Arcuate nucleus (ARC) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p=0.023$). Quantification of Fos positive cells revealed that Fos expression in the ARC of virgin rats after NPY was significantly greater (2-fold) than in the pregnant NPY group (Fig.6.11). I.c.v. injection of NPY significantly increased Fos expression in the virgin group ($p<0.001$) (20.9 ± 2.7 vs control 10.3 ± 0.7 Fos positive cells/ARC), with no effect in the pregnant group (Fig.6.11 and Fig.6.12).

Lateral Hypothalamic Area (LHA) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was not a statistically significant difference in Fos expression among virgin and pregnant groups (Fig.6.13). I.c.v. injection of NPY did not significantly increase Fos expression in either the virgin or the pregnant group. (Fig.6.13)

Ventro-medial Hypothalamus (VMH) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was not a statistically significant difference in Fos expression among virgin and pregnant groups (Fig.6.14). I.c.v. injection of NPY did not significantly increase Fos expression in either the virgin or the pregnant group (Fig.6.14).

Dorso-medial Hypothalamus (DMH) Fos expression

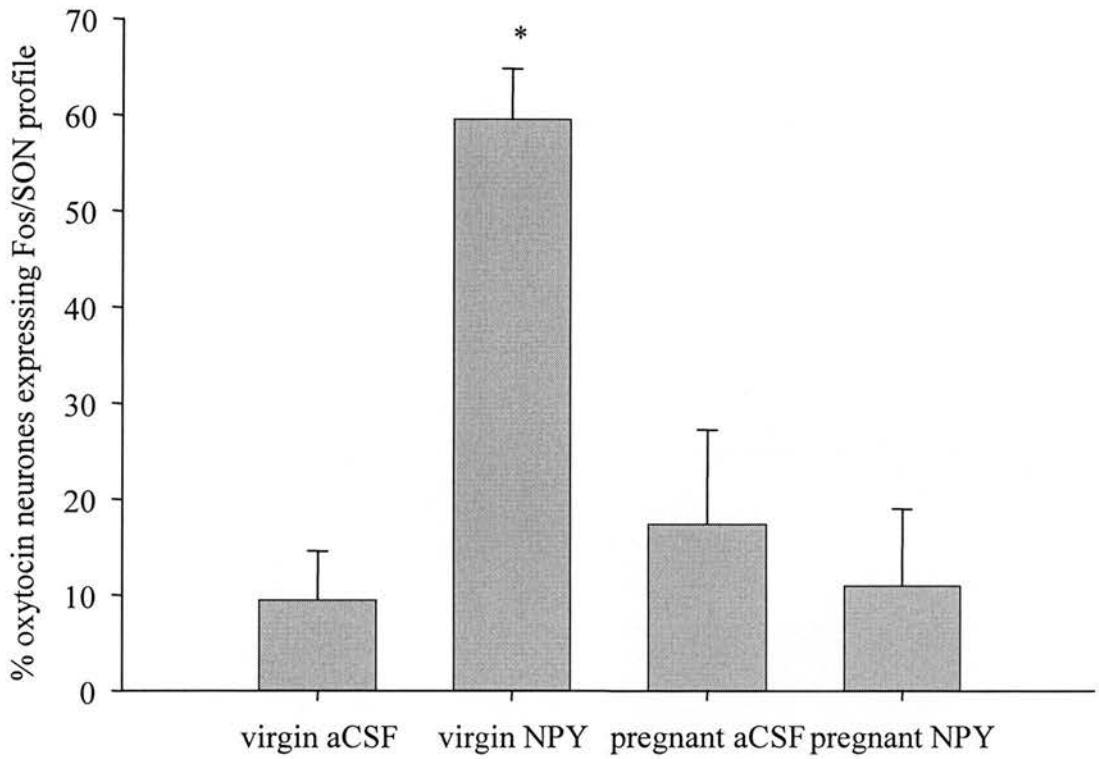


Figure 6.10: The effect of i.c.v. NPY on oxyotcin neurone activation in the SON in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 minutes post-injection of NPY. The number of oxytocin cells were counted and the number of oxytocin cells expressing Fos was divided by the total number and expressed as a percentage. Values are the group means ± SEM. Virgin/aCSF, n= 6; virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA Followed by a Student Newman Keuls multiple comparison test: *$p<0.002$ vs all other groups.

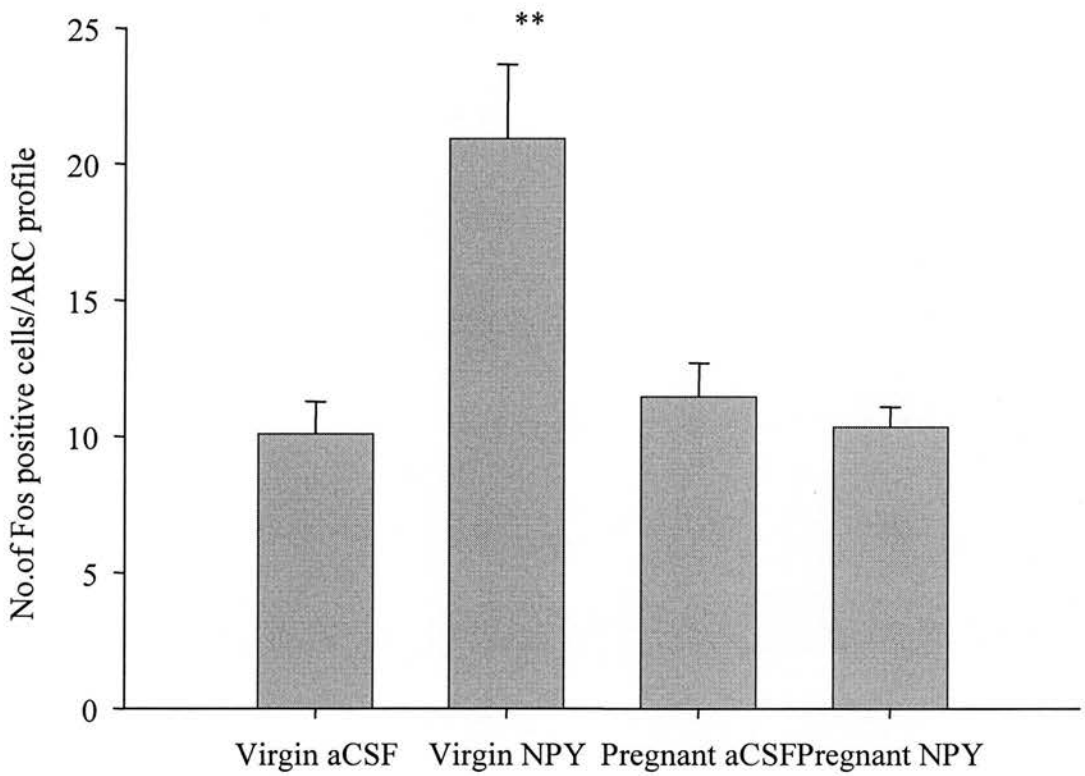


Figure 6.11: The effect of i.c.v. NPY on Fos positive cell counts in the ARC in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=6; virgin/ NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: **$p<0.001$ vs all other groups.

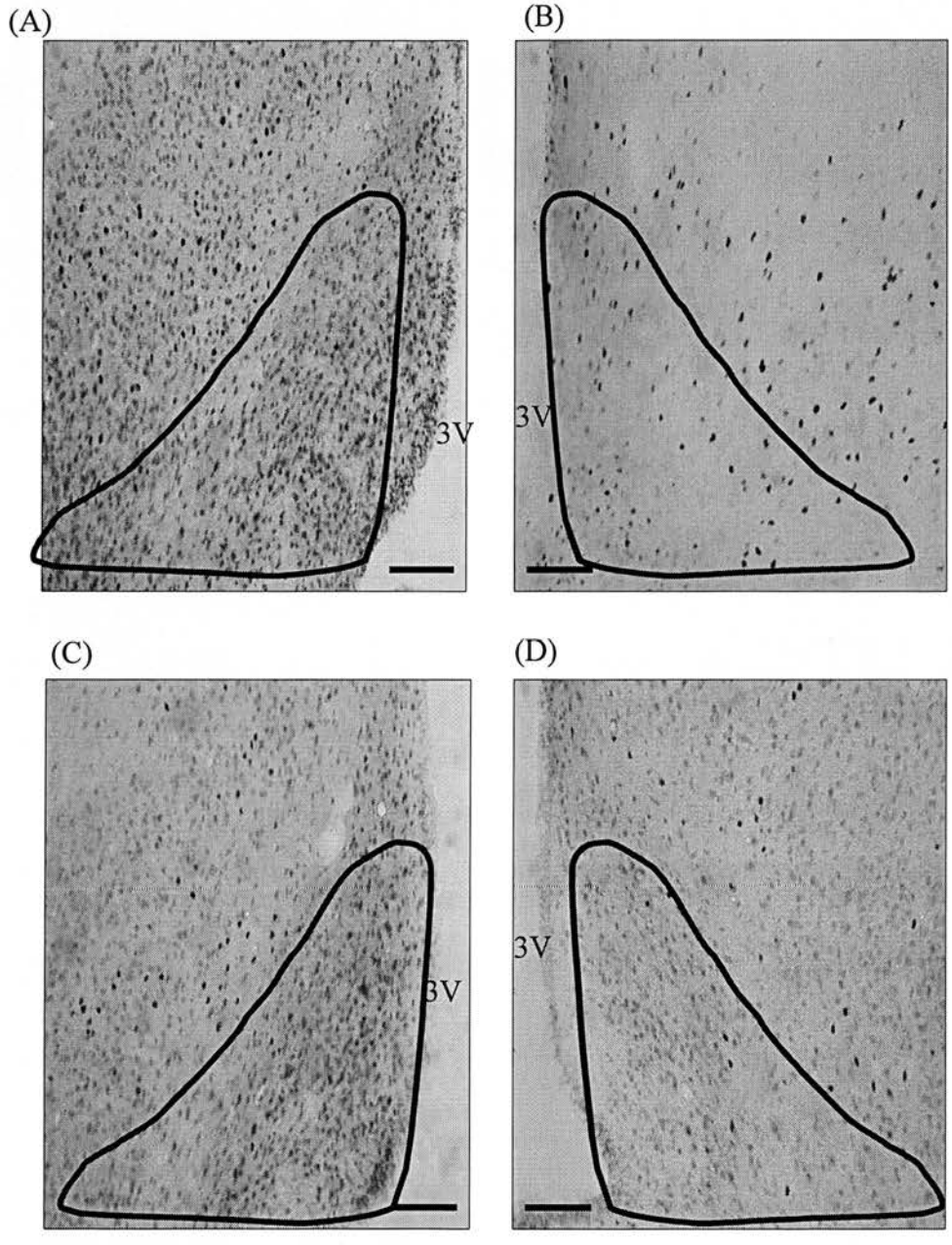


Figure 6.12: Representative photomicrographs of coronal sections through the ARC in virgin and pregnant rats. Sections were processed for Fos immunohistochemistry: (A) virgin aCSF; (B) virgin NPY; (C) pregnant aCSF; (D) pregnant NPY. Scale Bar = 100μm. 3V = 3rd ventricle.

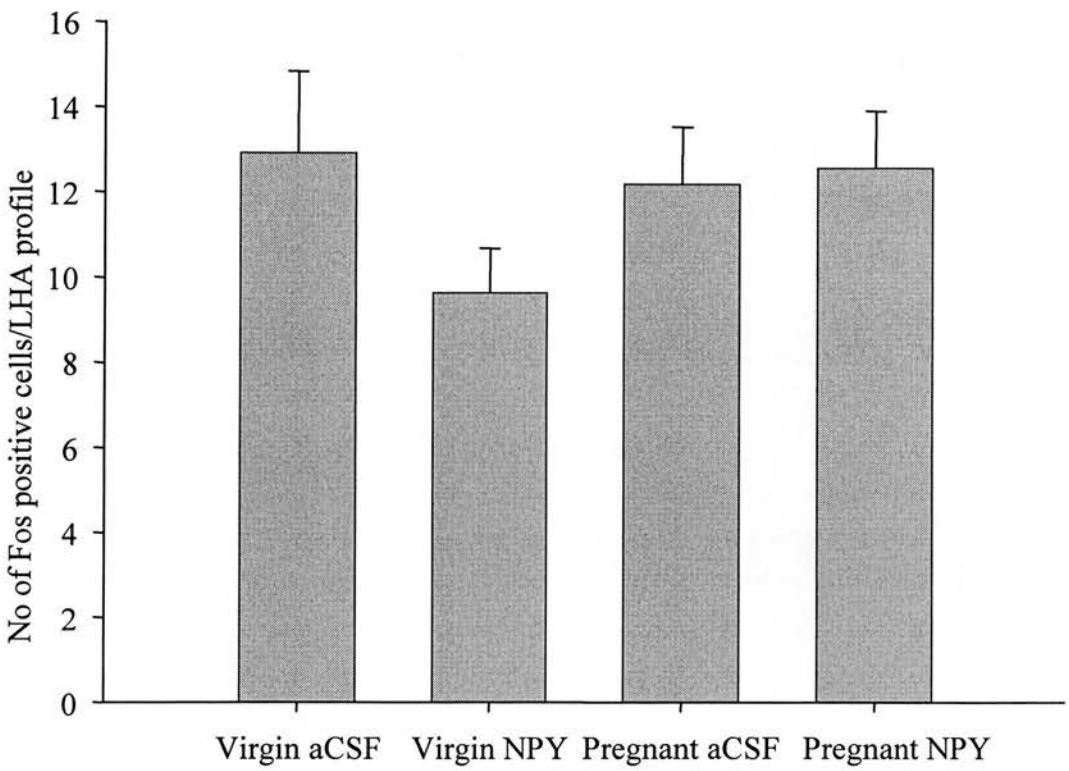


Figure 6.13: The effect of i.c.v. NPY on Fos positive counts in the LHA in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=6; virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA.

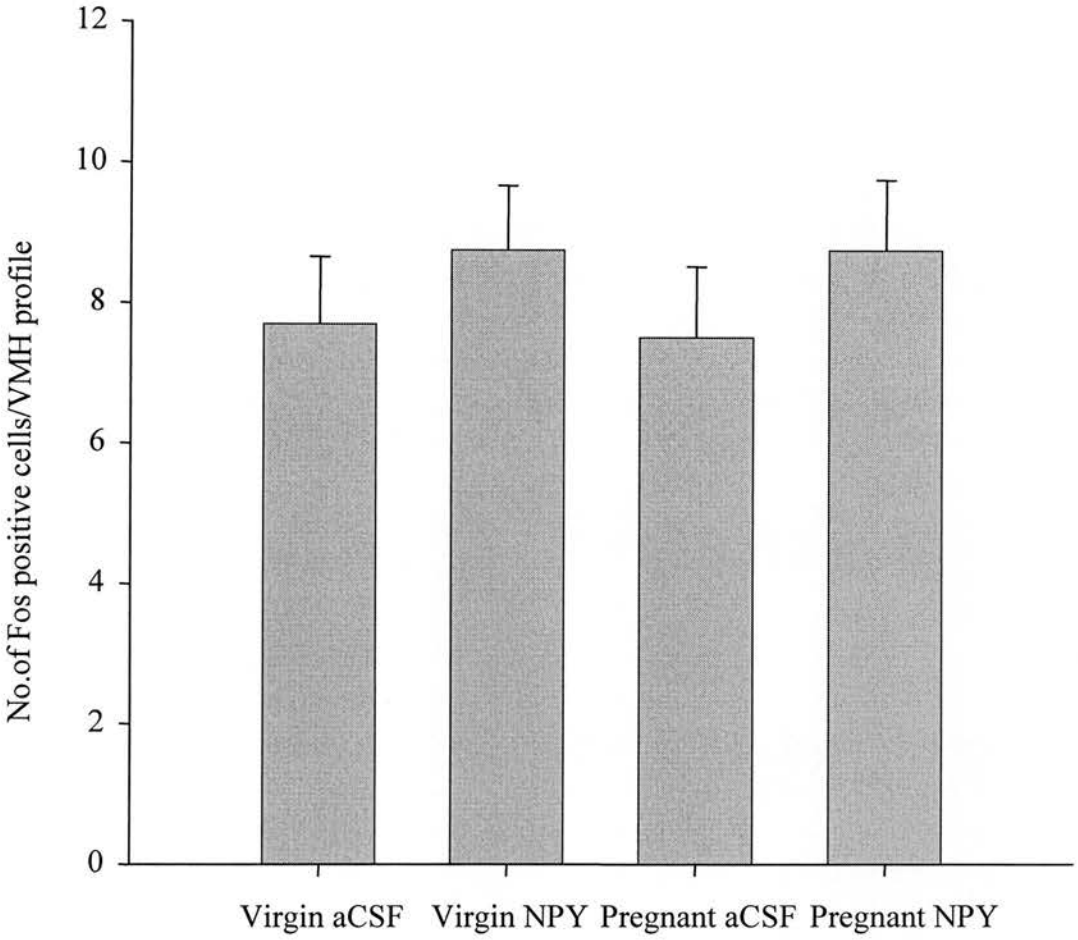


Figure 6.14: The effect of i.c.v. NPY on Fos positive cell counts in the VMH in virgin and pregnant rats. Rats were killed by transcardial perfusion 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin/aCSF, n=6; virgin/NPY, n=6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA.

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p=0.033$) (Fig.6.15). I.c.v. injection of NPY did not significantly increase Fos expression in either the virgin or the pregnant group (Fig.6.15). Quantification of Fos positive cells revealed that Fos expression in the DMH of pregnant rats was significantly greater (1.5-fold) than in the virgin aCSF group (Fig.6.15).

6.3.2 Experiment 2 – The effect of naloxone on reduced HPA responses to NPY in pregnancy

Blood glucose concentration

Analysis of plasma glucose concentration using a two-way RM ANOVA showed a statistically significant difference in plasma glucose concentration among virgin and pregnant groups ($p<0.001$). Basal blood glucose concentrations were significantly lower in pregnant rats than virgins ($p<0.001$; two-way RM ANOVA). NPY with i.v. saline significantly increased plasma glucose in the virgin group ($p< 0.001$; two-way RM ANOVA) within 15 min (7.8 ± 0.25 vs basal 6.7 ± 0.13 mmol/l) with glucose concentrations returning to basal by 30 min (Fig.6.16). I.c.v. injection of NPY with i.v. naloxone significantly increased plasma glucose in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min (8.0 ± 0.1 vs basal 5.8 ± 0.12 mmol/l) with glucose concentrations returning to basal by 30 min (Fig.6.16). I.c.v. injection of NPY and i.v. saline had no significant effect on blood glucose concentration in pregnant rats, nor did i.c.v. injection of NPY and i.v. naloxone. There were no significant differences in blood glucose concentration following i.v. naloxone or saline in either the virgin or the pregnant group (Fig 6.16).

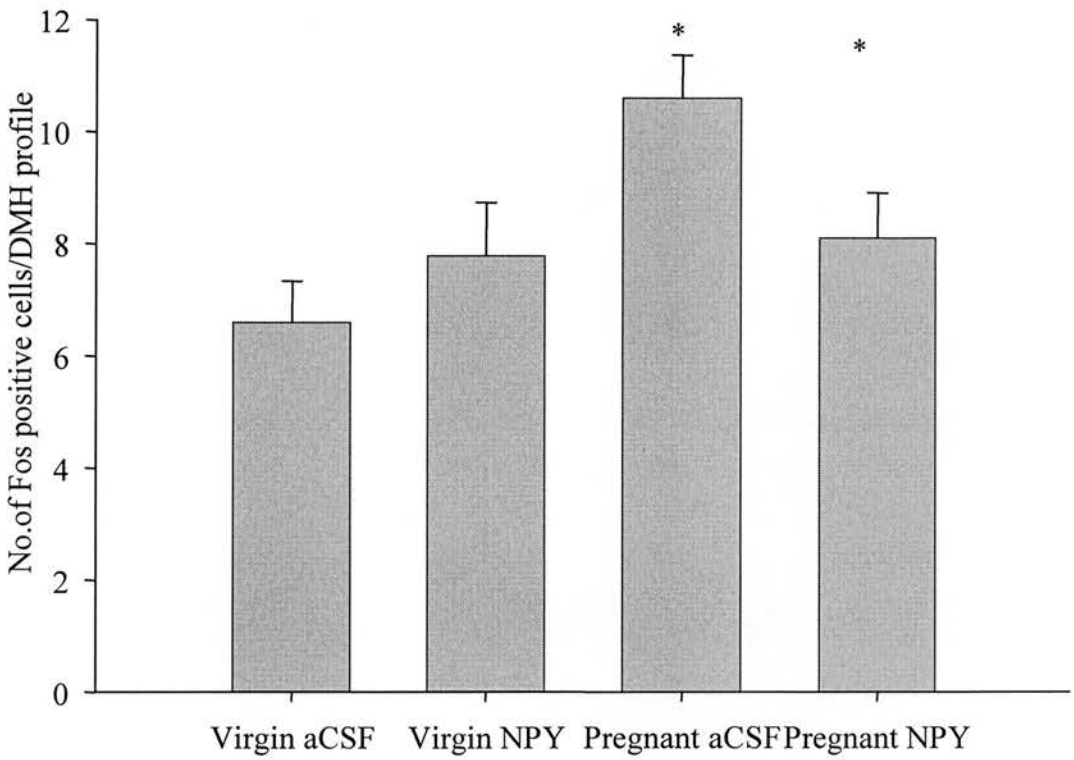


Figure 6.15: The effect of i.c.v. NPY on Fos positive cell counts in the DMH in virgin and pregnant rats. Rats were killed by transcardial perfusion 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three Sections and values are the group means ± SEM. Virgin/aCSF, n=6; virgin/NPY, n= 6; pregnant/aCSF, n=6; pregnant/NPY, n=5. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs virgin aCSF.

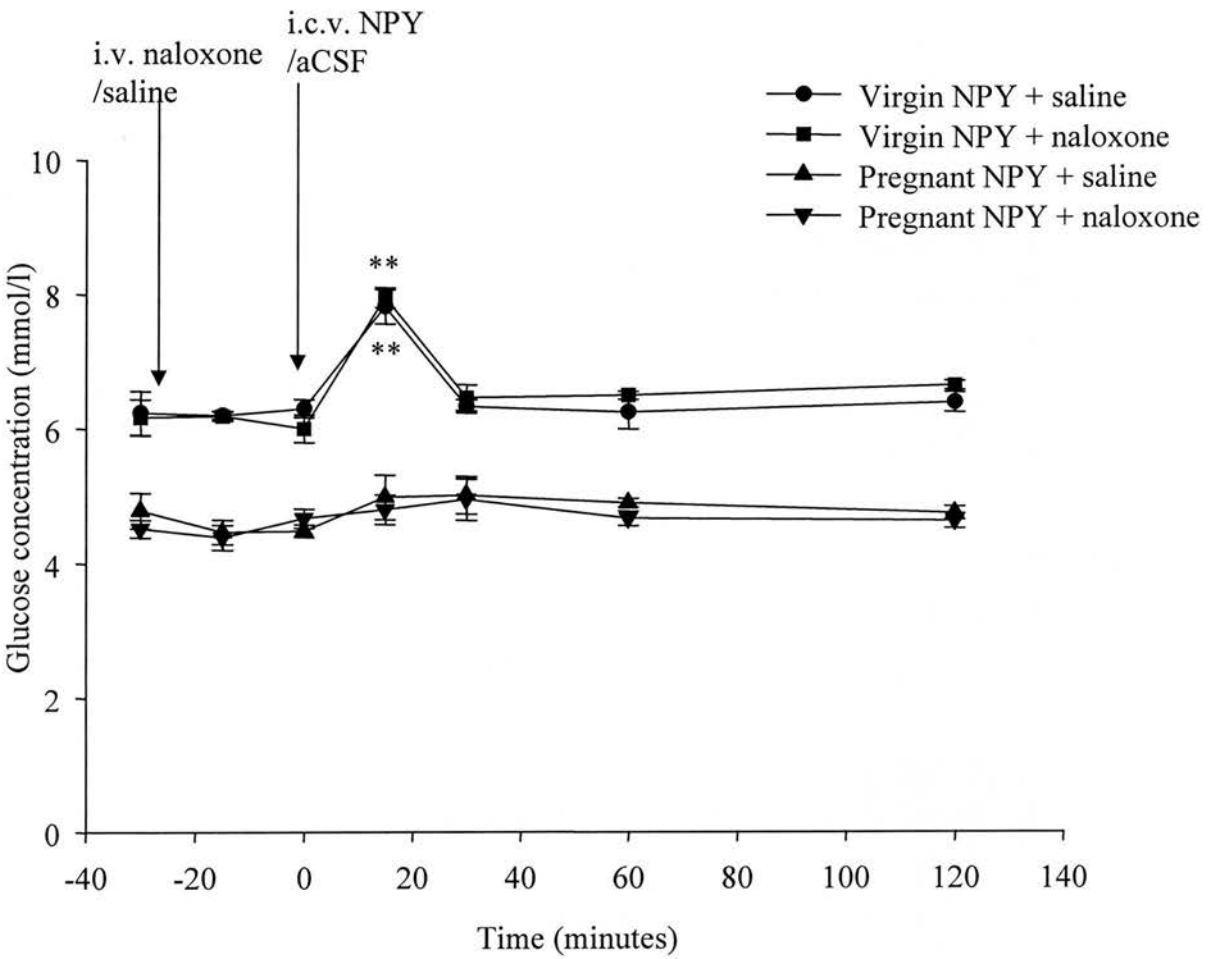


Figure 6.16: The effect of i.c.v NPY and i.v. naloxone on blood glucose concentration in virgin and pregnant rats. Three basal blood samples were taken 30, 15 and 1 min prior to i.c.v. NPY (5μg/rat). I.v. naloxone was given 30min prior to i.c.v. NPY. Further blood samples were withdrawn 15,30, 60 and 120 min post infusion. Values are group means ± SEM. Virgin NPY/saline, n=6; virgin NPY/naloxone, n=6; pregnant NPY/saline, n=6; pregnant NPY/naloxone, n=6. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests: **$p<0.001$ significantly different from basal.

Plasma ACTH concentration

Analysis of plasma ACTH concentration data using a two-way RM ANOVA showed there was a statistically significant difference in plasma ACTH concentrations among virgin and pregnant groups ($P<0.001$). Basal plasma concentrations of ACTH were not different among groups. I.c.v. injection of NPY and i.v. saline significantly increased plasma ACTH concentration in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min (214.8 ± 50.7 vs basal 54.8 ± 9.4 pg/ml) (Fig .6.17) with concentrations remaining significantly elevated for 30 min (Fig.6.17). I.c.v injection of NPY and i.v. naloxone significantly increased plasma ACTH concentration in the virgin group ($p<0.001$; two-way RM ANOVA) within 15 min (175.5 ± 35 vs basal 61.8 ± 14.3 pg/ml) (Fig.6.17) with concentrations remaining significantly elevated for 60 min (Fig.6.17). I.c.v NPY and i.v. saline had no significant effect on plasma ACTH in the pregnant group (Fig. 6.17). There was a tendency for plasma ACTH concentration to increase from basal in the pregnant group treated with i.v. naloxone ($p=0.059$; two-way RM ANOVA) within 15 min (128.7 ± 25 vs basal 42.7 ± 3.6 pg/ml), and ACTH concentration at this time was greater than in the pregnant group given NPY and i.v. saline, and not different from ACTH concentration in the virgin rats at this time after NPY (Fig.6.17). Analysis of changes in plasma ACTH concentration from basal at 15 min using a two-way ANOVA showed there was a statistically significant difference in plasma ACTH concentration among virgin and pregnant groups ($p = 0.023$), and that the ACTH concentration in the pregnant rats given naloxone and NPY was not different from that in virgin rats, but was greater than in the pregnant rats given NPY and saline (Fig.6.18). Basal ACTH concentration are the values before NPY.

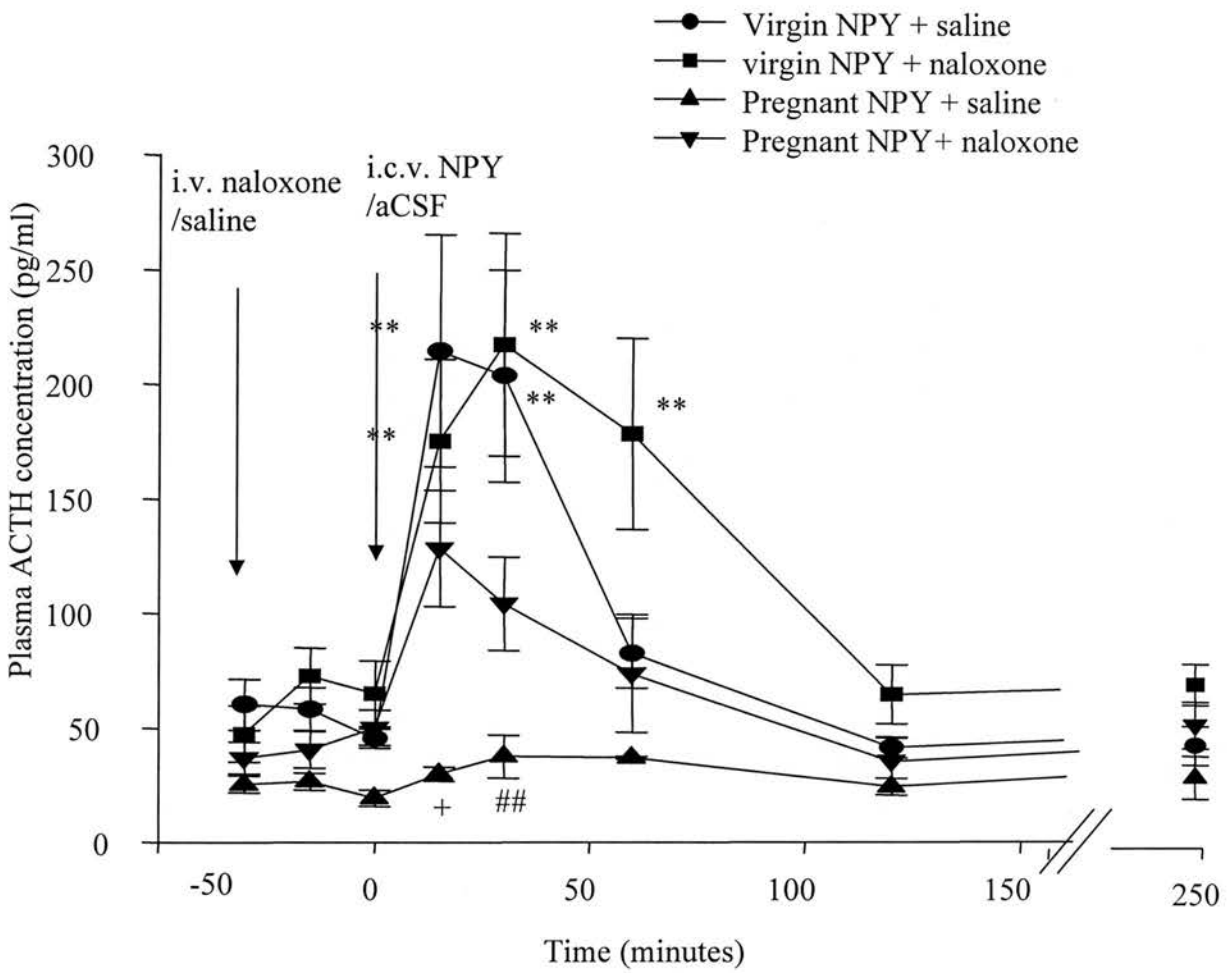


Figure 6.17: The effect of i.c.v. NPY and i.v. naloxone on plasma ACTH concentration in virgin and pregnant rats. Three basal blood samples were taken 30, 15 and 1 min prior to i.c.v. NPY. Further blood samples were withdrawn 15, 30, 60 and 120 min post-infusion. Trunk blood was collected at 240 min. Values are group means ± SEM. Virgin NPY/saline, n=6; virgin NPY/naloxone, n=6; pregnant NPY/saline, n=6; pregnant NPY/naloxone, n=6. Two-way ANOVA for repeated measures followed by Student Newman Keuls multiple comparison tests: **$p<0.001$, significantly different from basal. + $p<0.05$ significantly different from all other groups at the same time point.

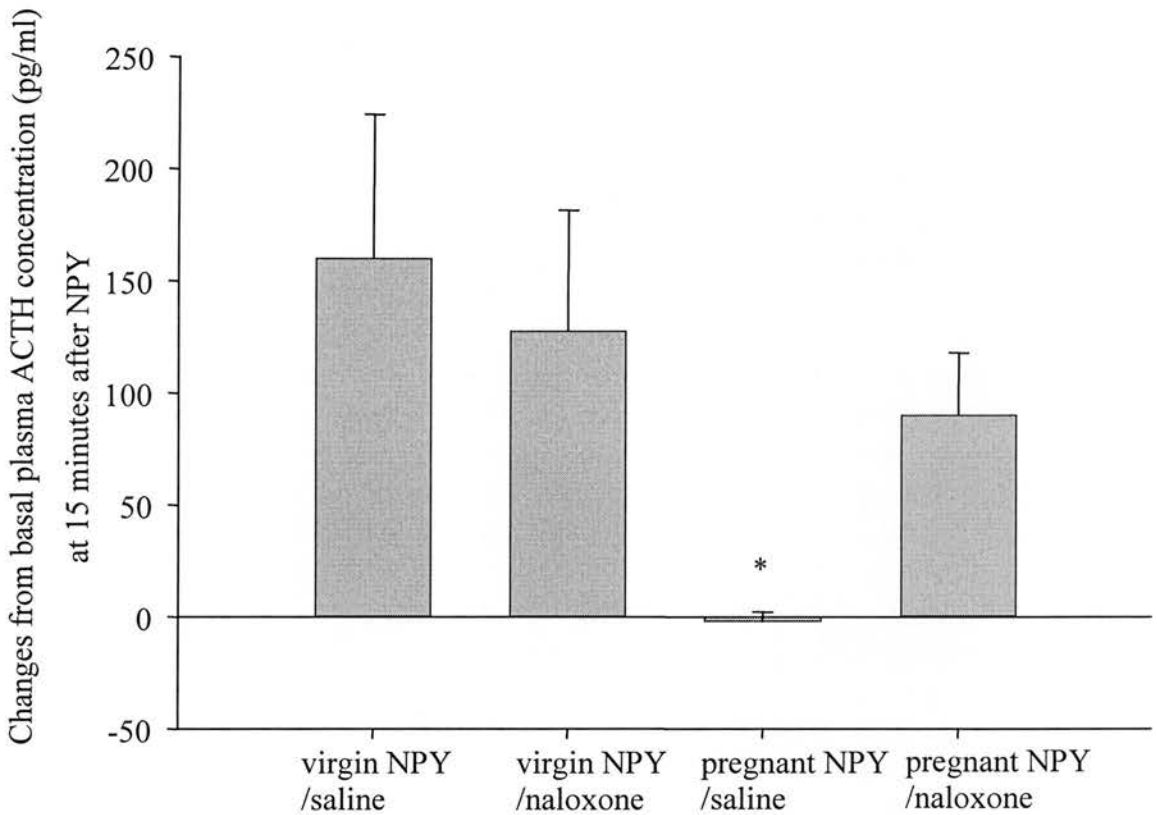


Figure 6.18: The effect of i.c.v NPY and i.v. naloxone on plasma ACTH concentration in virgin and pregnant rats. Delta values for the measurements at 30 minutes after NPY subtracted from the first basal blood sample were calculated. Values are the measurements at 30 minutes after NPY subtracted from the first basal blood sample Were calculated. Values are the group means ± SEM. Virgin NPY/saline, n=6; virgin NPY/naloxone, n=6; pregnant NPY/saline, n=6; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison Test: *$p<0.05$ vs all other groups.

CRH mRNA expression in the parvocellular PVN

Analysis of CRH mRNA ISH data using a two-way ANOVA showed there was a statistically significant difference in CRH mRNA expression in the pPVN among virgin and pregnant groups ($p = 0.046$). Quantification of the number of CRH positive cells revealed that CRH mRNA expression in the dorsomedial parvocellular subdivision of the PVN was significantly greater (1.5-fold) in virgin rats given NPY and saline than in the pregnant group given NPY and saline (Fig. 6.19). I.c.v injection of NPY and naloxone significantly increased CRH mRNA expression in the pregnant group ($p = 0.041$) compared with i.c.v. injection of NPY and saline (Fig 6.19).

AVP mRNA in the parvocellular PVN

Analysis of AVP mRNA ISH data using a two-way ANOVA showed there was a statistically significant difference in AVP mRNA grain area (mm^2) in the pPVN among virgin and pregnant groups ($p=0.003$). Quantification of grain area revealed that AVP mRNA expression in the parvocellular area of the PVN was significantly greater in virgin rats given i.c.v NPY and i.v. saline (10-fold) than in the pregnant NPY-treated group given saline (Fig 6.20 and Fig 6.21). I.c.v. injection of NPY with i.v. naloxone significantly increased AVP mRNA expression in the pregnant group ($p=0.004$) compared with i.c.v. injection of NPY with i.v. saline, with no significant effect of naloxone in the virgin rats (Fig 6.20 and Fig 6.21).

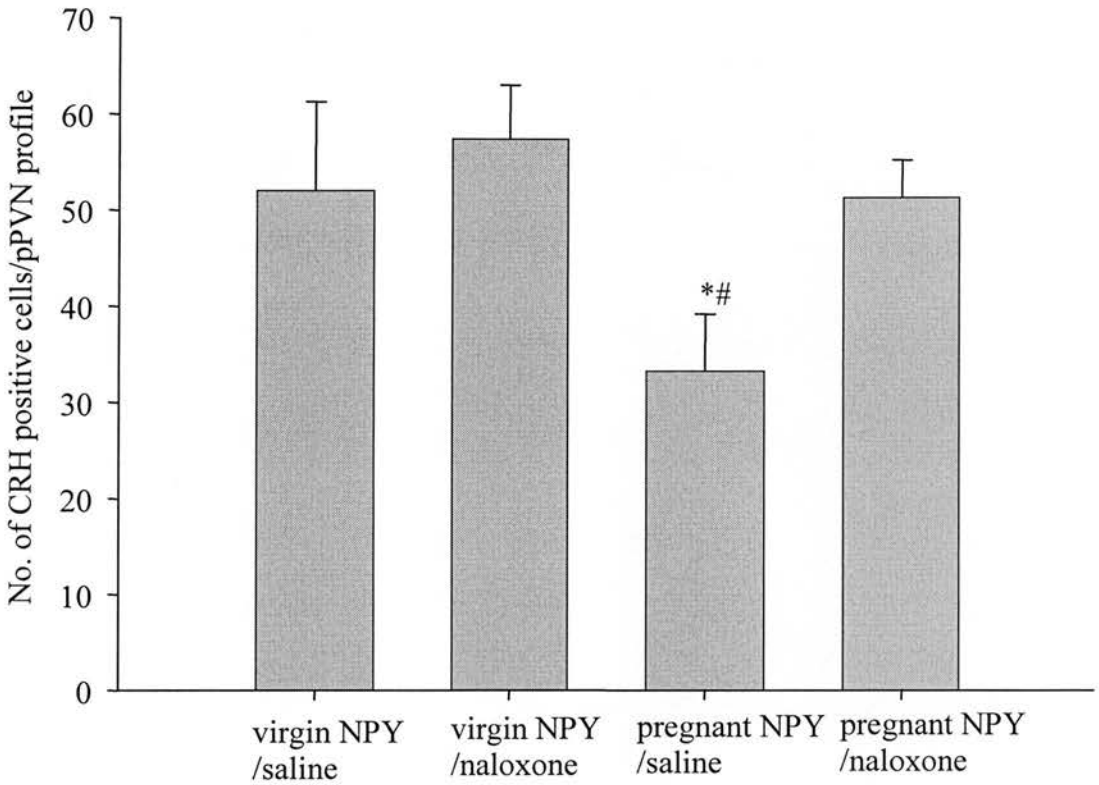


Figure 6.19: The effect of i.c.v. NPY and i.v. naloxone on CRH mRNA expression in the pPVN in virgin and pregnant rats. I.v. naloxone was given 30 min prior to i.c.v. NPY (5μg/rat). Rats were killed 240 min after NPY. Values are group means ± SEM. Virgin NPY/saline, n=6; virgin NPY/naloxone, n=6; pregnant NPY/saline, n=6; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs pregnant NPY/naloxone; #$p<0.05$ vs virgin NPY/saline.

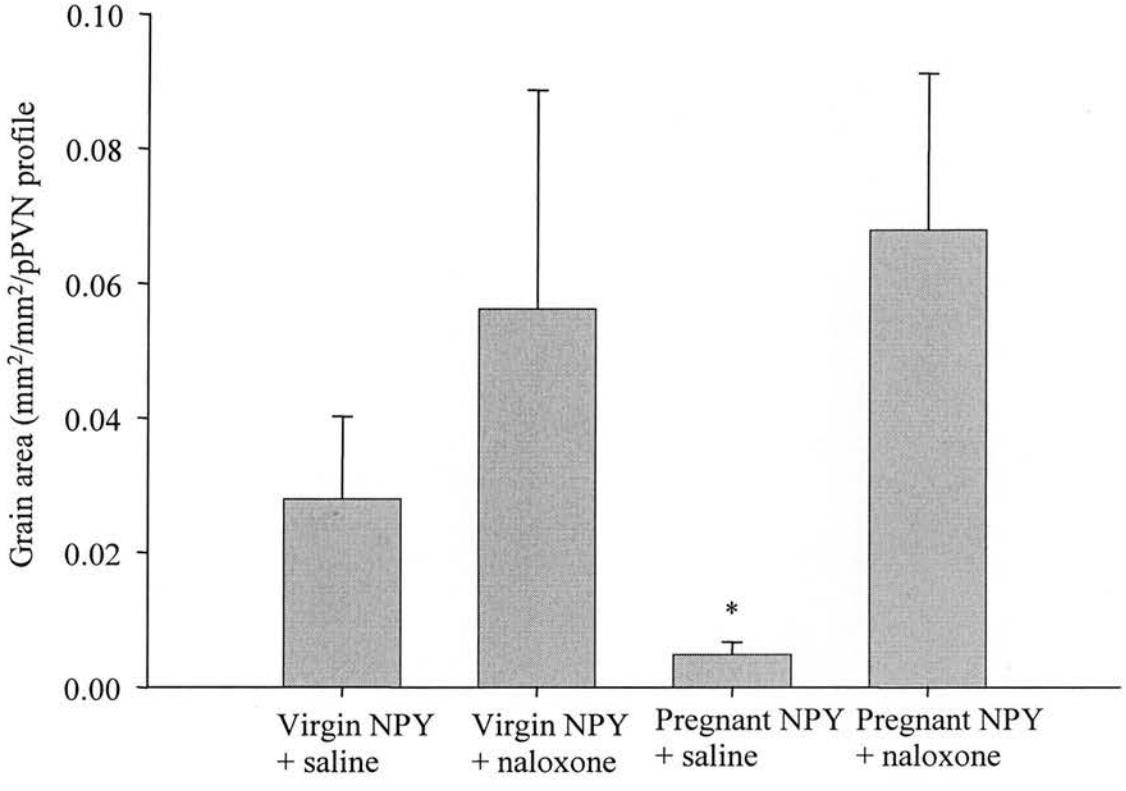


Figure 6.20: The effect of i.c.v. NPY and i.v. naloxone on AVP mRNA (mm^2/mm^2) in the pPVN in virgin and pregnant rats. I.v. naloxone was given 30 min prior to i.c.v. NPY (5μg/rat). Rats were killed 240 min after NPY. Values are group means ± SEM. Virgin NPY/saline, n=6; virgin NPY/naloxone, n=6; pregnant NPY/saline, n = 6; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs all other groups.

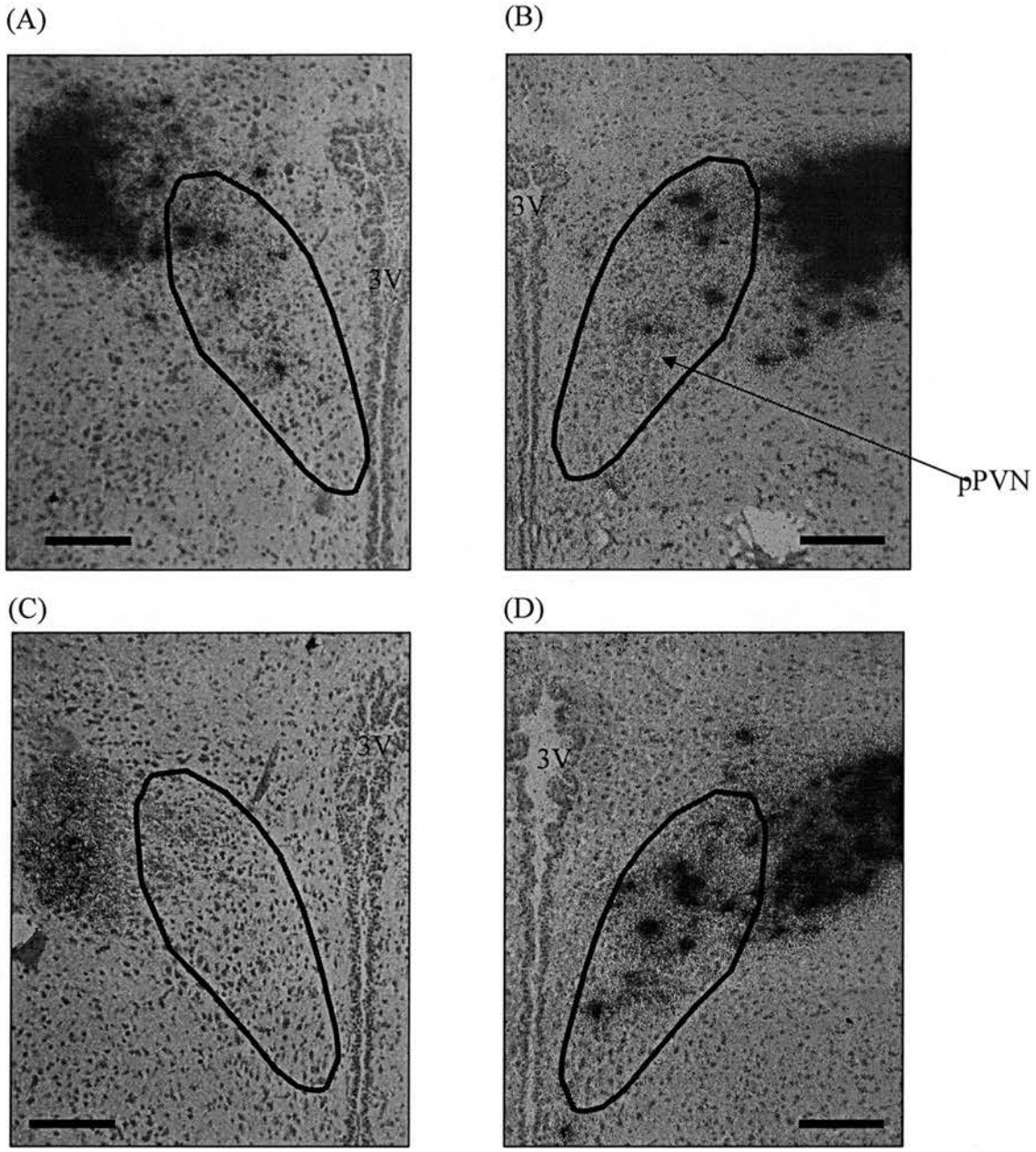


Figure 6.21: Representative photomicrographs of coronal sections through the PVN hybridised with a ^{35}S labelled probe complementary to AVP mRNA:
(A) virgin NPY/saline; (B) virgin NPY/naloxone; (C) pregnant NPY/saline; (D) pregnant NPY/naloxone. Scale bar = 100μm. 3V = 3rd ventricle. pPVN = parvocellular PVN

6.3.3 Experiment 3 – Fos expression in response to i.c.v. NPY after administration of naloxone

Behavioural Data

Analysis of behavioural data using a two-way ANOVA showed a statistically significant difference among groups in eating behaviour following NPY administration ($p=0.003$). Behavioural observations showed that NPY significantly increased eating behaviour in both virgin and pregnant rats given saline ($p=0.017$; two-way ANOVA) (Fig.6.22) or naloxone ($p=0.043$; two-way ANOVA) (Fig.6.22). There were no significant differences between rats given NPY and naloxone vs rats given NPY and saline in either the virgin or the pregnant group although naloxone showed a tendency to decrease eating in all groups; eating behaviour after NPY was lower in the pregnant/ naloxone treated group, than in the virgin/ naloxone (Fig.6.22). There were no statistically significant differences in drinking between groups, except that drinking behaviour was significantly less in the pregnant group given NPY+ naloxone than in the pregnant group given NPY+ saline (Fig 6.22). There were no significant differences in grooming between groups, except that grooming was significantly less in the pregnant group given NPY+ naloxone than in the pregnant group given NPY+ saline (Fig 6.22).

Analysis of food weight data using a two-way ANOVA showed a statistically significant difference in food weights following NPY administration ($p<0.001$). NPY significantly reduced food weight in virgin and pregnant rats ($p=0.003$; two-way ANOVA) given saline (Fig 6.23). In virgin and pregnant rats given NPY and naloxone ($p=0.042$) food weight was significantly reduced in only the virgin rats (Fig 6.23).

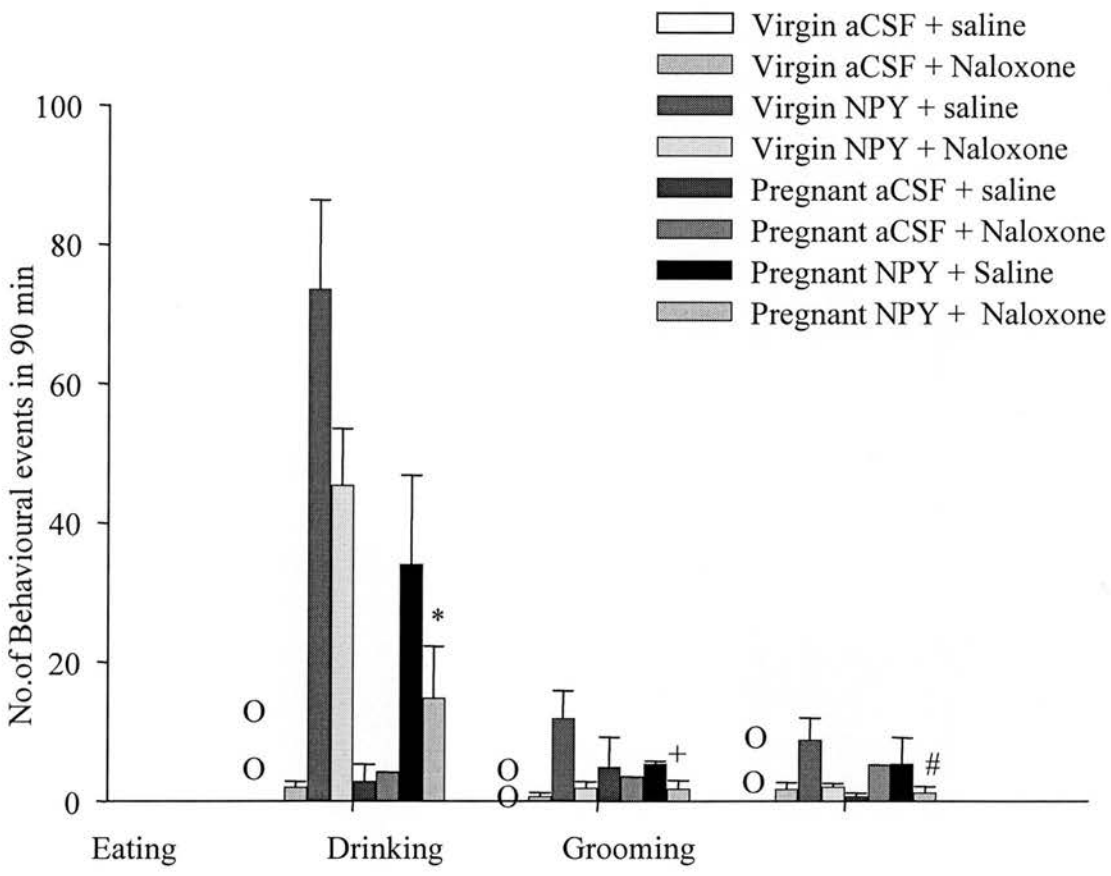


Figure 6.22: The effect of i.c.v. NPY and i.v. naloxone on behaviours in virgin and pregnant rats. Values are the group means ± SEM. Virgin aCSF/saline, n=2 (values shown as circles); virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/ naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n=5; pregnant NPY/saline, n=5; pregnant NPY/naloxone, n=6/ Two-way ANOVA followed by Stduent Newman Keuls multiple comparison tests excluding virgin aCSF /saline: *$p<0.05$ vs virgin NPY/naloxone; +$p<0.05$ vs NPY/saline; #$p<0.051$ vs NPY/saline.

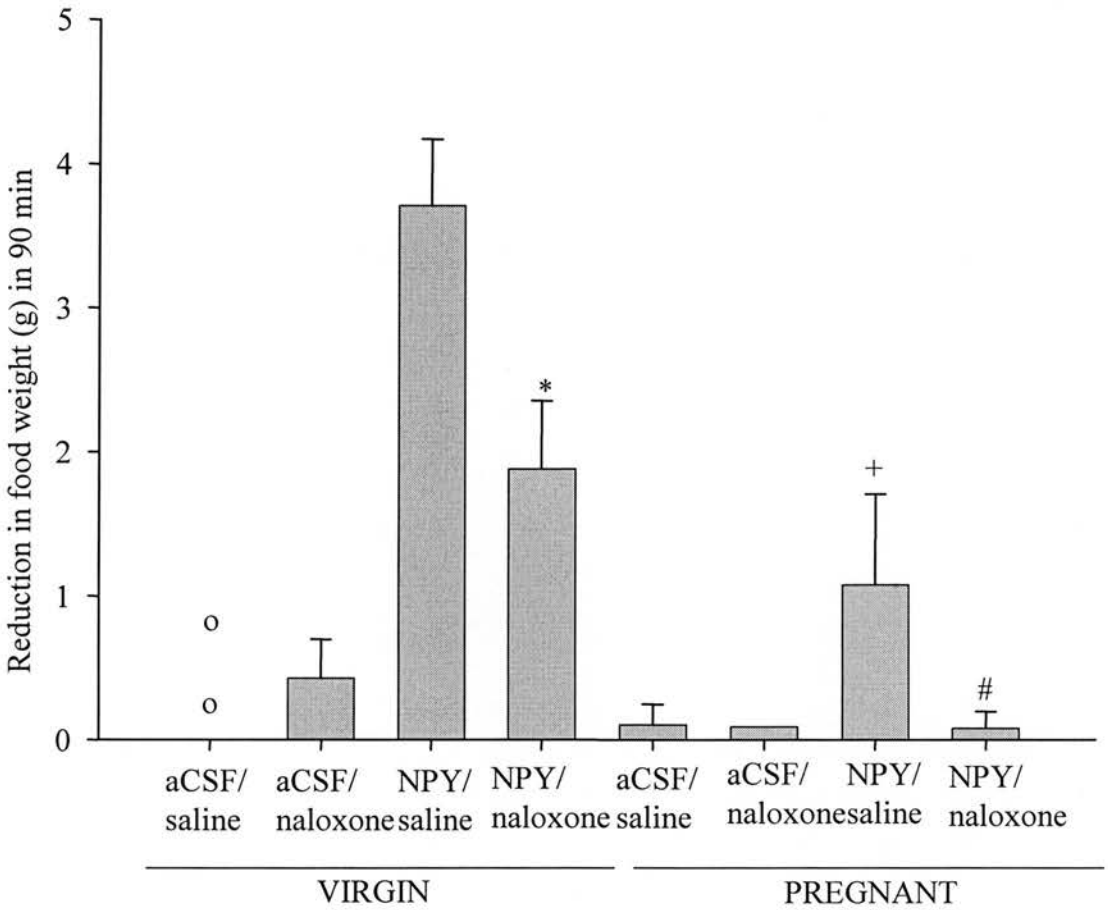


Figure 6.23: The effect of i.c.v. NPY and i.v. naloxone on food intake in virgin and pregnant rats. Values are the group means ± SEM. Virgin aCSF/saline, n=2 (values shown as circles); virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n=5; pregnant NPY/saline, n=5; pregnant NPY/naloxone, n=6. Two-way ANOVA Followed by Student Newman Keuls multiple comparison tests excluding virgin aCSF/saline: significant effect of pregnancy, $p<0.001$ of naloxone $p=0.003$; *$p=0.022$ vs virgin NPY/naloxone; +$p<0.033$ vs virgin NPY/saline; #$p<0.001$ vs virgin NPY/naloxone.

Analysis of the time taken to start eating using a two-way ANOVA showed a statistically significant difference following NPY administration between virgin and pregnant rats (p=0.018). Pregnant rats given NPY and naloxone took significantly longer to begin eating than virgin rats given NPY and naloxone (p=0.027) (Fig 6.24). Pregnant rats given NPY and naloxone also took significantly longer to begin eating than pregnant rats given NPY and saline (p=0.025) (Fig 6.24).

Parvocellular paraventricular nucleus (PVN) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups (p = 0.006). Quantification of Fos positive cells revealed that Fos expression in the pPVN of virgin rats given NPY and saline was significantly greater than in the pregnant NPY + saline group (P<0.001). Fos expression in the PVN of virgin rats given NPY and saline was 3.5-fold greater than in the pregnant NPY group given saline (Fig.6.25). I.c.v. injection of NPY and i.v. saline significantly increased Fos expression in the virgin group (p=0.002) (26.5 ± 5.6 vs control 7.6 ± 0.6 Fos positive cells/PVN), but not in the pregnant rats (Fig.6.25 and Fig. 6.26).

Quantification of Fos positive cells revealed that Fos expression in the PVN of virgin rats given NPY and naloxone was not significantly greater than in the pregnant NPY and naloxone group (Fig.6.25). I.c.v. injection of NPY and i.v. naloxone significantly increased Fos expression in the virgin group (p=0.031) (20.0 ± 4.5 vs control 8.2 ± 0.6 Fos positive cells/PVN) (Fig.6.25 and Fig.6.26). I.c.v. injection of NPY and i.v. naloxone significantly increased Fos expression in the pregnant group (p=0.025) (13.8 ± 1.4 vs respective control (aCSF + naloxone 5.1 ± 0.12 Fos positive cells/PVN) (Fig.6.25 and Fig.6.26).

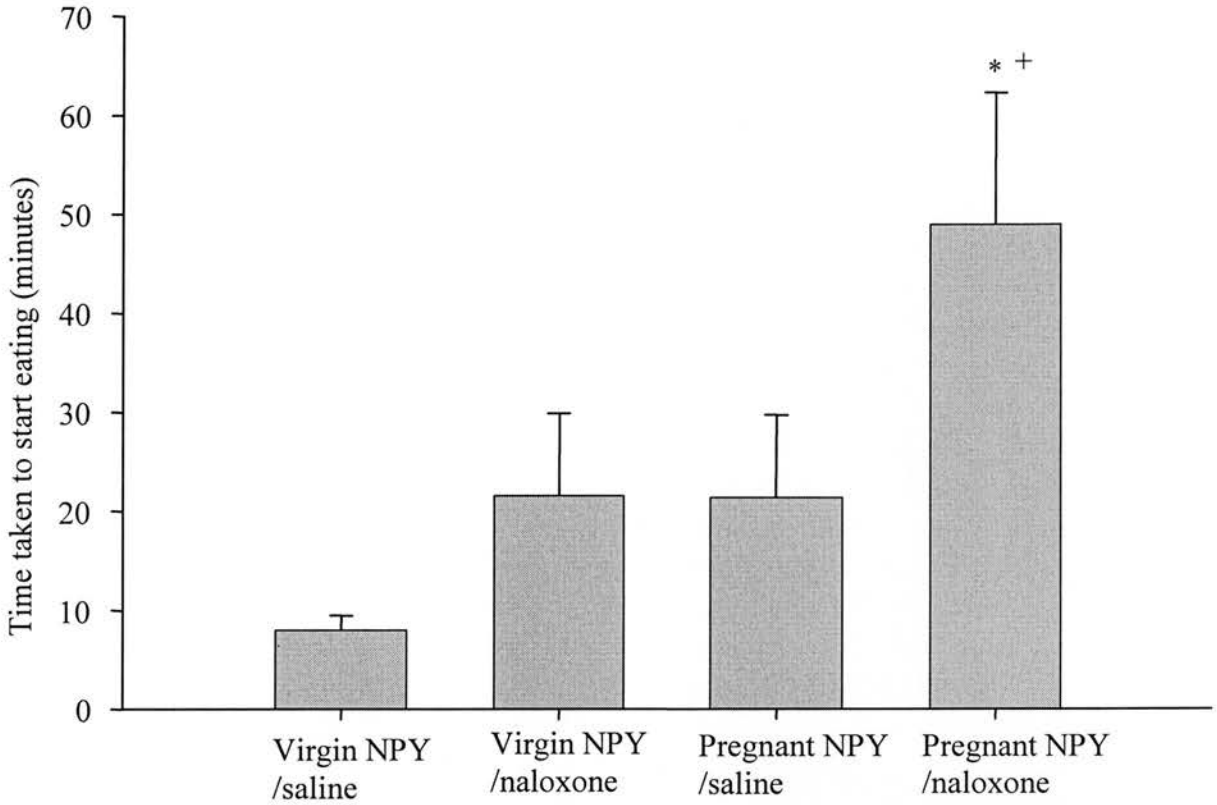


Figure 6.24: The effect of i.c.v NPY and i.v. naloxone on the time taken to begin eating in virgin and pregnant rats. Virgin NPY/saline, n=7; virgin NPY /naloxone, n=6; pregnant NPY/saline, n=5; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by Student Newman Keuls multiple comparison Tests: *$p<0.05$ vs virgin; +$p<0.05$ vs pregnant NPY/saline. Values are the group means ± SEM.

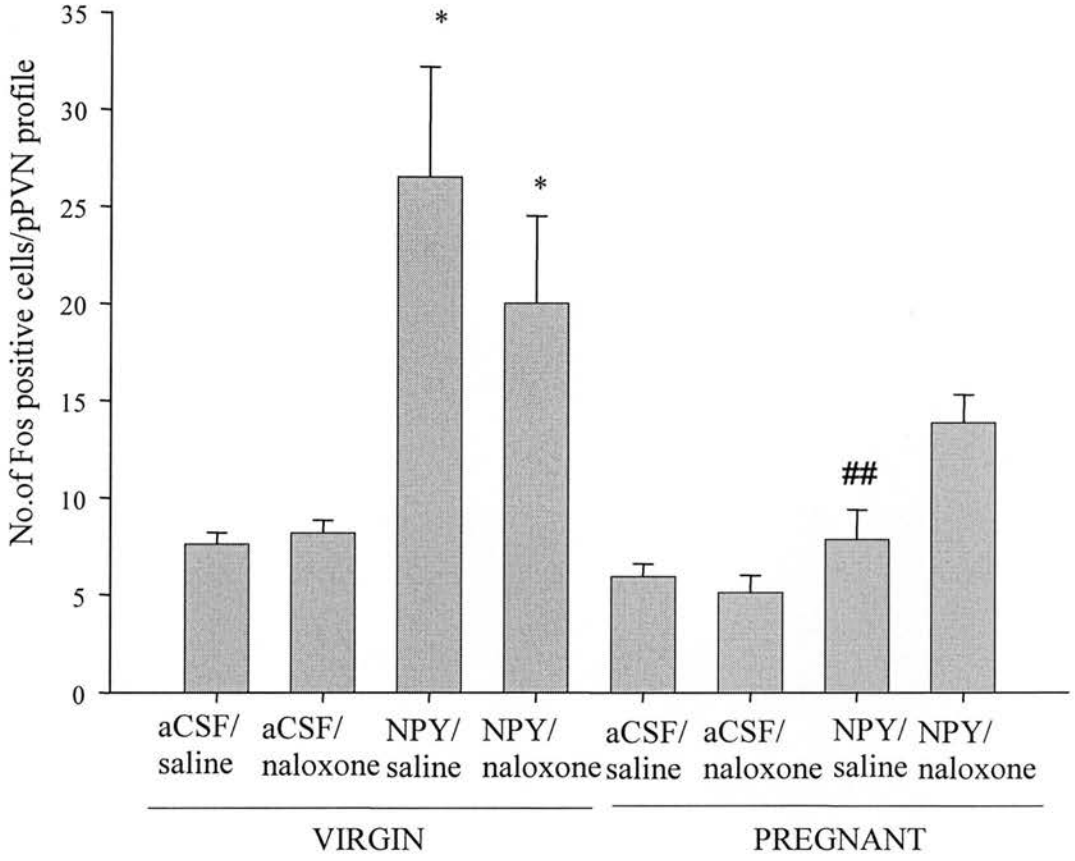


Figure 6.25: The effect of i.c.v NPY and i.v. naloxone on Fos positive cell counts in the pPVN in virgin and pregnant rats. Rats were killed by transcardial perfusion 90 minutes post-injection of NPY. Values are the mean count positive cells over three sections and values are the group means ± SEM. Virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n=5; pregnant NPY/saline, n=5; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test. *$p<0.05$ vs aCSF; ##$p<0.001$ vs virgin NPY/saline.

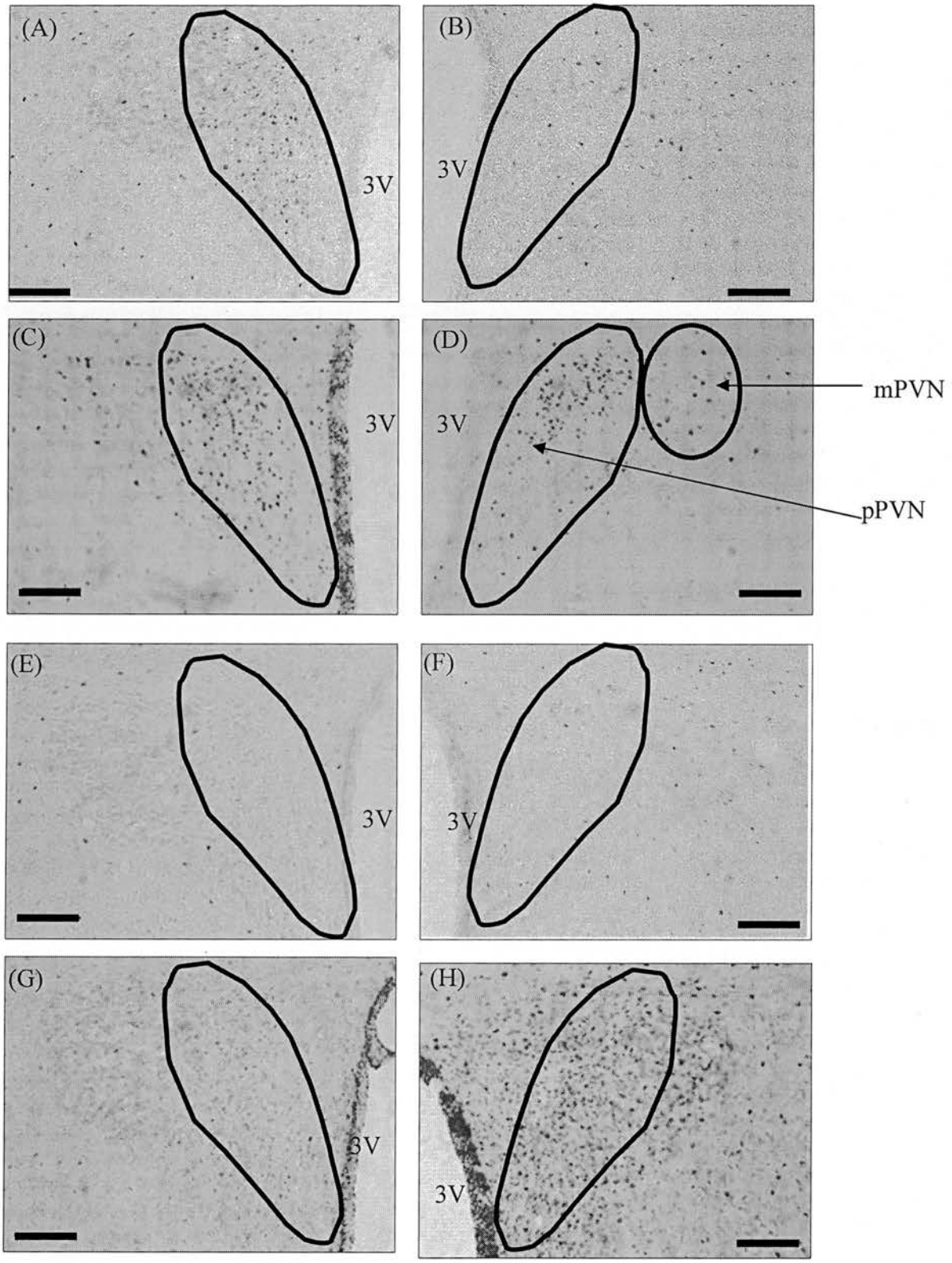


Figure 6.26: Representative photomicrographs of coronal sections through the PVN in virgin and pregnant rats. Sections were processed for Fos Immunohistochemistry: (A) virgin aCSF/saline; (B) virgin aCSF/naloxone; (C) virgin NPY/saline; (D) virgin NPY/naloxone; (E) pregnant aCSF/saline; (F) pregnant aCSF/naloxone; (G) pregnant NPY/saline; (H) pregnant NPY/ naloxone. Scale bar = 100μm. 3V= 3rd ventricle.

Magnocellular paraventricular nucleus Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($P = 0.003$). Quantification of Fos positive cells revealed that Fos expression in the mPVN of virgin rats given NPY and saline was significantly greater (2-fold) than in the pregnant NPY + saline group ($P<0.001$). I.c.v. injection of NPY and i.v. saline significantly increased Fos expression in the virgin group ($p<0.001$) (17.8 ± 4.7 vs control 4.7 ± 0.4 Fos positive cells/mPVN) (Fig.6.27 and Fig.6.26). Quantification of Fos positive cells revealed that Fos expression in the mPVN of virgin rats given NPY and naloxone was significantly greater (2-fold) than in the pregnant NPY + naloxone group ($P=0.003$). I.c.v. injection of NPY and i.v. naloxone significantly increased Fos expression in the virgin group ($p=0.012$) (16.2 ± 2.7 vs control 16.2 ± 1.2 Fos positive cells/PVN) (Fig.6.27and Fig. 6.26). In pregnant rats, Fos expression in the mPVN was greater in the group given NPY and naloxone than in the group given aCSF and naloxone (Fig 6.26).

Supraoptic Nucleus (SON) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p=0.007$). Quantification of Fos positive cells revealed that Fos expression in the SON of virgin rats given NPY and saline was significantly greater (2-fold) than in the pregnant NPY and saline group ($p=0.05$). I.c.v. injection of NPY and i.v. saline significantly increased Fos expression in the virgin group ($p=0.009$) (18.6 ± 4.6 vs control 5.8 ± 0.8 Fos positive cells/SON) (Fig.6.28). Quantification of Fos positive cells revealed that there were no significant differences between virgin rats

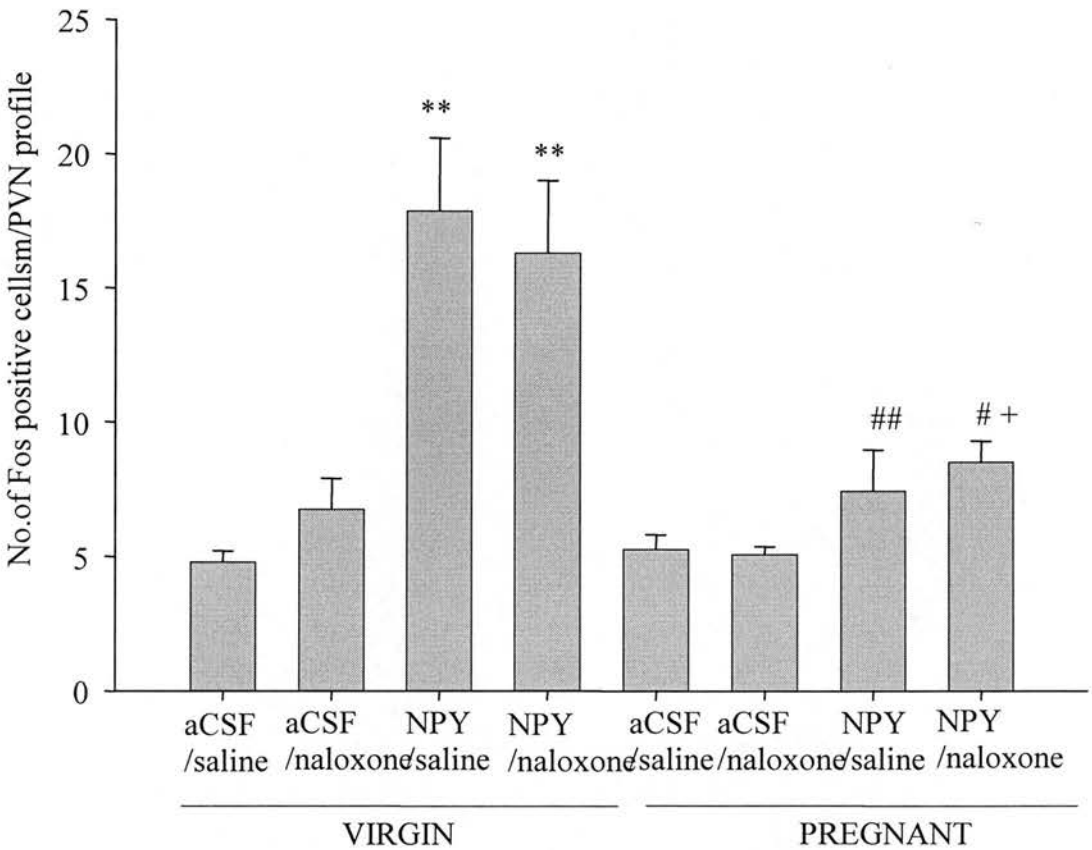


Figure 6.27: The effect of i.c.v. NPY and i.v. naloxone on Fos positive cell counts in the mPVN in virgin and pregnant rats. Rats were killed by transcardial Perfusion fixation 90 minutes post injection of NPY. Values are the mean count positive cells over three sections and values are the group means ± SEM. virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n = 5; pregnant NPY/saline, n=5; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: **p<0.001 vs aCSF saline and naloxone; ##p<0.001 vs virgin aCSF/saline, #p<0.05 vs virgin aCSF/naloxone. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test amongst pregnant: +p<0.05 vs aCSF/naloxone.

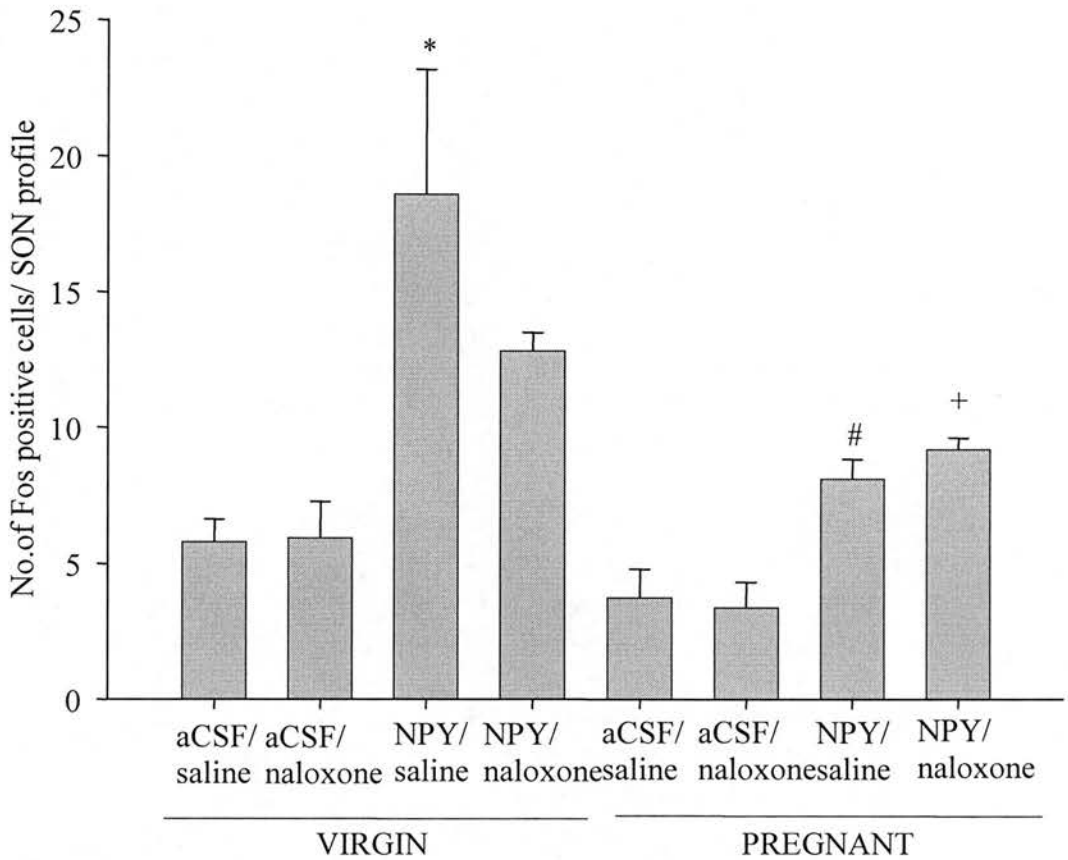


Figure 6.28: The effect of i.c.v. NPY and i.v. naloxone on Fos positice cell counts in the SON in virgin and pregnant rats. Rats were killed by transcardial Perfusion fixation 90 minutes post-injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. Virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; Virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n=5; pregnant NPY/saline, n=5; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: *$p<0.05$ vs virgin aCSF groups, #$p<0.05$ vs virgin NPY/saine; +$p<0.05$ vs pregnant aCSF/naloxone.

given NPY and naloxone and pregnant rats given NPY and naloxone (Fig.6.28). Fos expression was greater in pregnant rats given NPY and naloxone than in pregnant rats given aCSF and naloxone (Fig 6.28).

Arcuate nucleus (ARC) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p<0.001$). Quantification of Fos positive cells revealed that Fos expression in the ARC of virgin rats given NPY and saline was significantly greater (1.5-fold) than in the pregnant NPY and saline group ($p<0.001$). I.c.v. injection of NPY and i.v. saline significantly increased Fos expression in the virgin group ($p<0.001$) (11.3 ± 0.9 vs control 5.3 ± 1.3 Fos positive cells/ARC) (Fig.6.29).

Quantification of Fos positive cells revealed that Fos expression in the ARC of virgin rats given NPY and naloxone was significantly greater (1.5-fold) than in the pregnant NPY and naloxone group ($p<0.001$). I.c.v. NPY and i.v. naloxone significantly increased Fos expression in the virgin group ($p<0.001$) (11.6 ± 0.8 vs control 6.9 ± 0.6 Fos positive cells/ARC) (Fig.6.29). However, naloxone had no effect on responses to NPY in either virgin or pregnant rats.

Lateral hypothalamic area (LHA) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was not a statistically significant difference in Fos expression among virgin and pregnant groups (Fig. 6.32) I.c.v. injection of NPY did not significantly increase Fos expression in either the virgin or pregnant group under any treatment (Fig.6.30).

Ventromedial Hypothalamus (VMH) Fos expression

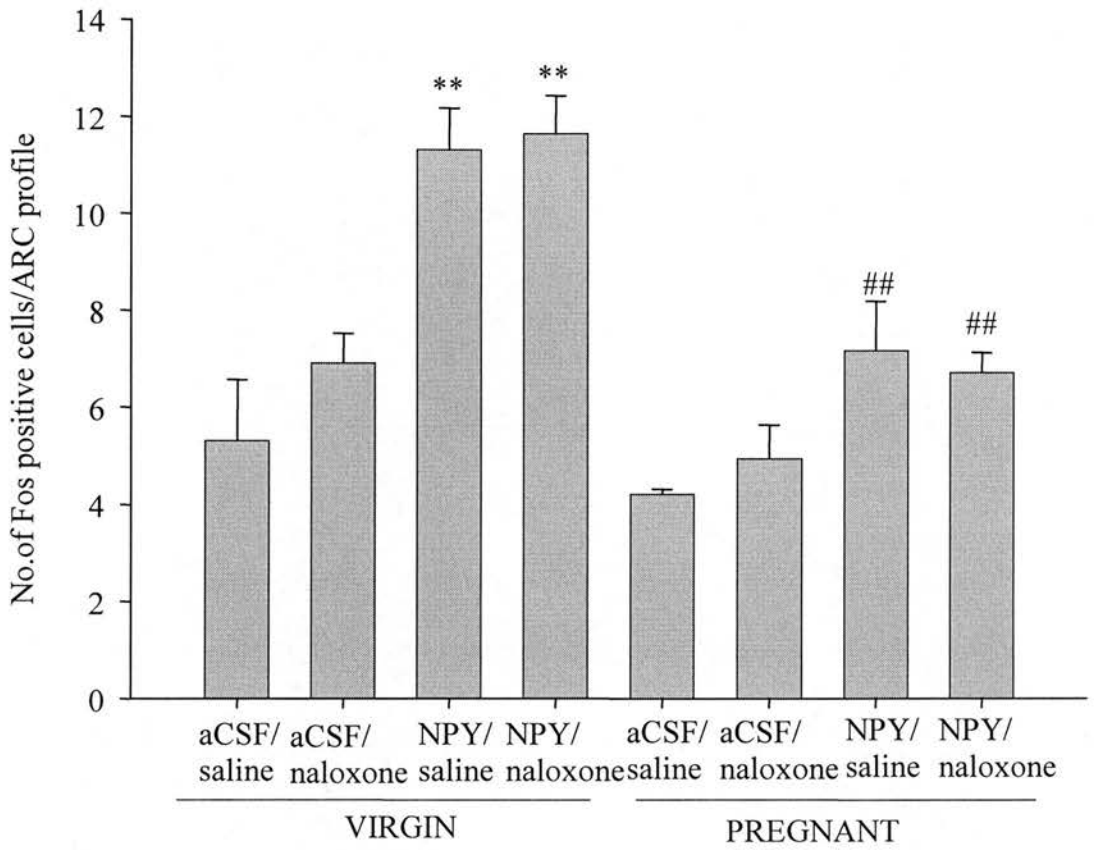


Figure 6.29: The effect of i.c.v. NPY and i.v. naloxone on Fos positive cell counts in the ARC in virgin and pregnant rats. Rats were killed by transcardial perfusion Fixation 90 minutes post-injection of NPY. Values are the mean count positive cells over three sections and values are the group means ± SEM. Virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n=5; pregnant NPY/ saline,n=5; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test: **$p<0.001$ vs aCSF; ##$p<0.001$ vs virgin NPY groups.

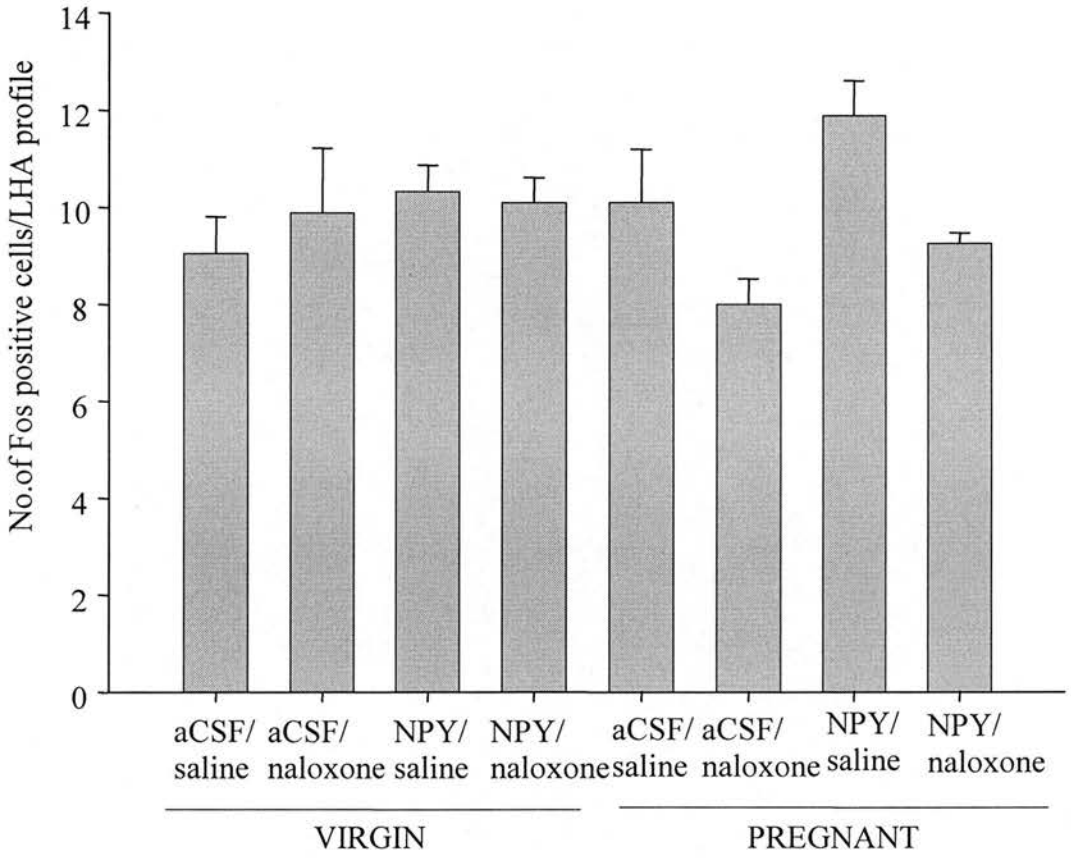


Figure 6.30: The effect of i.c.v. NPY and i.v. naloxone on Fos positive cell counts in the LHA in virgin and pregnant rats. Rat were killed by transcardial perfusion 90 minutes post-injection of NPY. Values are the mean count positive cells over three sections and values are the group means ± SEM. Virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n=5; pregnant NPY/saline, n= 5; pregnant NPY/naloxone, n=6. Two-way ANOVA showed no significant differences.

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p=0.008$). Quantification of Fos positive cells revealed that Fos expression in the VMH of virgin rats given aCSF and naloxone was significantly greater (1.5-fold) than in the pregnant aCSF and naloxone group ($p=0.024$). I.c.v. injection of NPY did not significantly increase Fos expression in either the virgin or the pregnant group under any treatment, and naloxone had no effect (Fig.6.31).

Dorsal Medial Hypothalamus (DMH) Fos expression

Analysis of Fos expression using a two-way ANOVA showed there was a statistically significant difference in Fos expression among virgin and pregnant groups ($p<0.001$).

Quantification of Fos positive cells revealed that Fos expression in the DMH of virgin rats given aCSF and naloxone was significantly greater (1.5-fold) than in the pregnant aCSF and naloxone group ($p=0.005$). I.c.v. injection of NPY did not significantly increase Fos expression in either the virgin or the pregnant group under any treatment (Fig.6.32).

6.4 Discussion

6.4.1. The effect of i.c.v. NPY on food intake

We have shown that NPY significantly increases eating behaviour and food intake in both virgin and pregnant female rats (Fig. 6.2 and Fig. 6.23). This corresponds with previous studies shown in male (Levine *et al,* 1984) and female rats (Brunton *et al,* 2006). Naloxone reduced NPY-induced feeding behaviour in both virgin and pregnant rats although not significantly (Fig 6.22). However, pregnant rats given

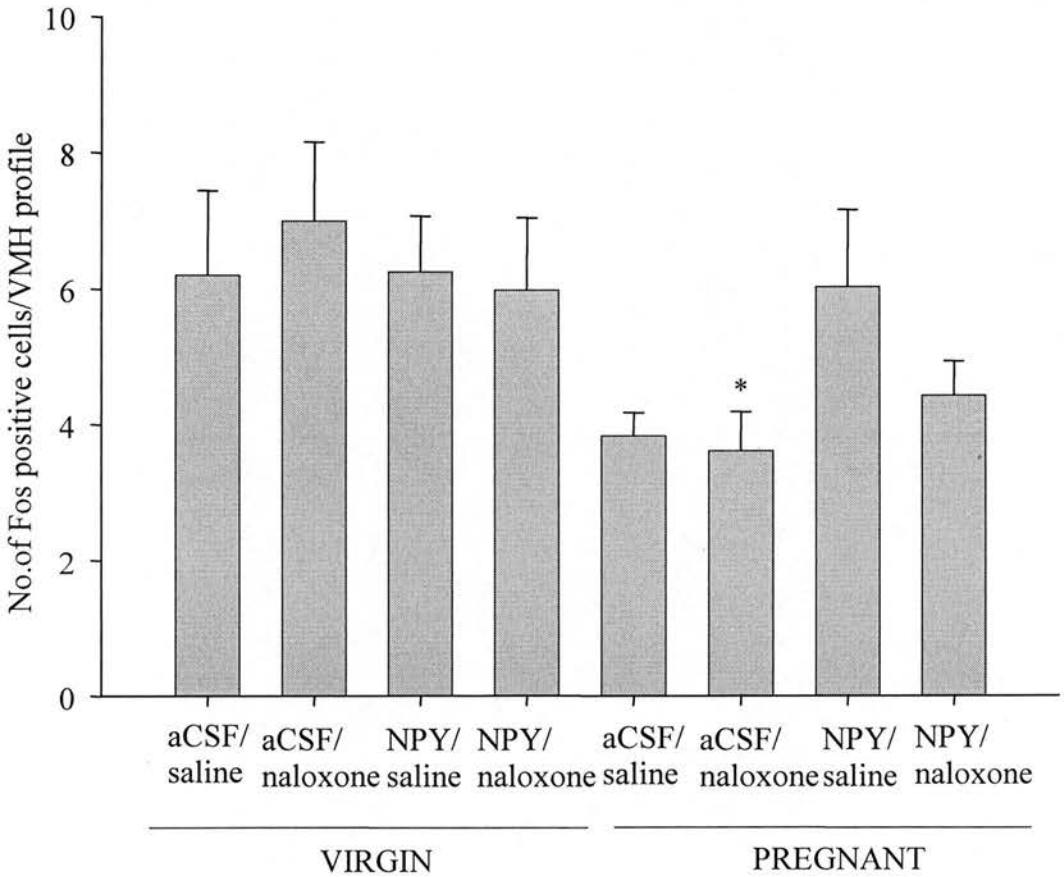


Figure 6.31: The effect of i.c.v. NPY and i.v. naloxone on Fos positive cell counts in the VMH in virgin and pregnant rats. Rats were killed by transcardial perfusion fixation 90 minutes post injection of NPY. Values are the mean counts of positive cells over three sections and values are the group means ± SEM. virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, n= 5; pregnant NPY/saline, n=6; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test. *$p<0.05$ vs virgin aCSF/naloxone.

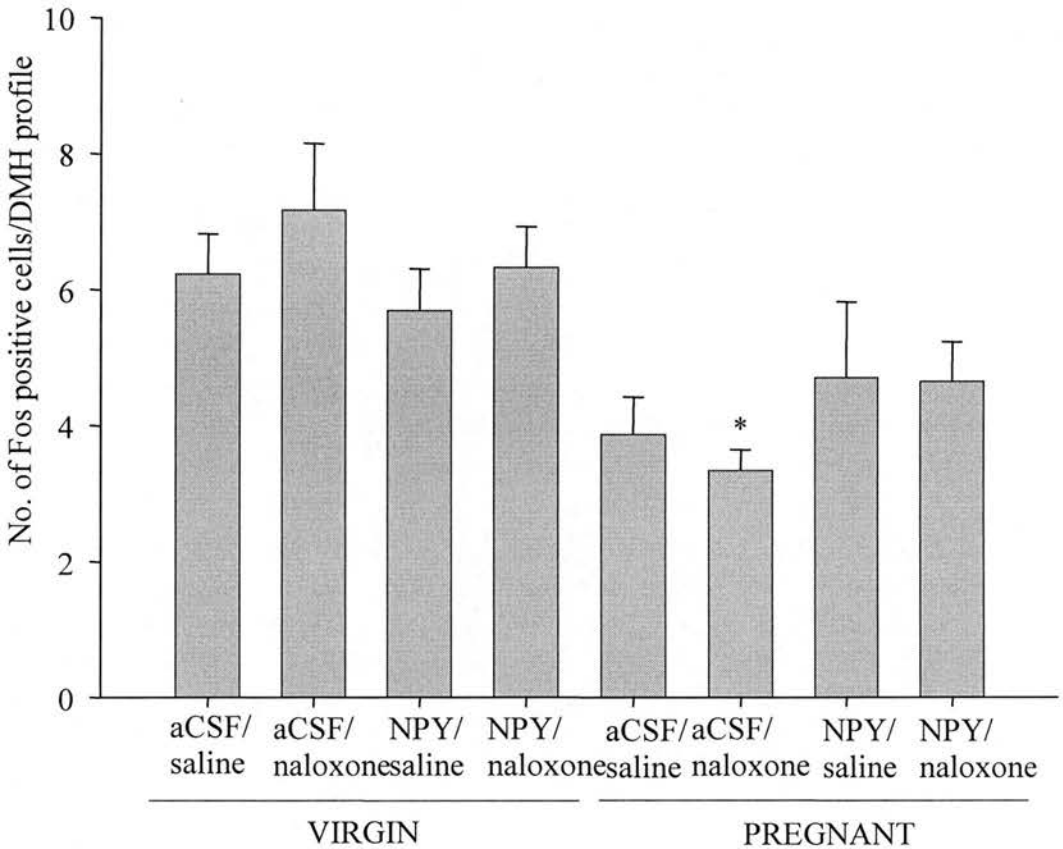


Figure 6.32: The effect of i.c.v. NPY and i.v. naloxone on Fos positive cell counts in the DMH in virgin and pregnant rats. Rats were killed by transcardial Perfusion 90 minutes post injection of NPY. Values are the mean counts of Positive cells over three sections and values are the group means ± SEM. Virgin aCSF/saline, n=4; virgin aCSF/naloxone, n=4; virgin NPY/saline, n=7; virgin NPY/naloxone, n=6; pregnant aCSF/saline, n=5; pregnant aCSF/naloxone, =5; pregnant NPY/saline, n=6; pregnant NPY/naloxone, n=6. Two-way ANOVA followed by a Student Newman Keuls multiple comparison test. *$p<0.005$ vs virgin aCSF.

NPY and naloxone ate significantly less than virgin rats given NPY and naloxone, and took much longer to initiate feeding behaviour (Fig 6.23 and Fig. 6.24). These findings are consistent with other evidence that endogenous opioid peptides stimulate feeding (Baile *et al,* 1986). Administration of naltrexone (another opioid receptor antagonist) into the NTS decreased feeding induced by NPY injected into the PVN, indicating that feeding produced by NPY in the PVN relies on functional opioid receptor pathways within the NTS (Kotz *et al,* 1998). It is thought that feeding signals arising from stimulation of NPY receptors in the PVN result in opioid release from the NTS, in particular the medial region (Kotz *et al,* 2000), and blockade of these opioid receptors prevents binding and action of the released opioids. In the present study naloxone reduced the NPY-induced increase in food intake as measured by a reduction in food weight (Fig 6.23). In virgin rats naloxone reduced the NPY-induced food intake by 60% yet in pregnant rats naloxone reduced the NPY-induced food intake by 90% (Fig.6.23). During pregnancy it has been shown that endogenous opioid mechanisms in the brain are up-regulated (Brunton *et al,* 2005; Douglas *et al,* 1998). The greater reduction in food intake after naloxone before NPY in late pregnant rats than in virgins is consistent with an important role in regulation of appetite of up-regulated opioid mechanisms in the brain in pregnancy. Pregnant rats given NPY and naloxone also took significantly longer to begin eating than the virgin rats given NPY and naloxone (Fig 6.24). Hence, endogenous opioids facilitated NPY-induced feeding in both virgin and late pregnant rats, but are evidently more important in late pregnant rats. NPY did not significantly increase drinking in any group. This is consistent with previous studies in male rats (Stanley *et al,* 1985) although one study suggested that NPY will increase drinking in

male rats (Clark *et al,* 1985). Grooming was increased by i.c.v. NPY only in virgin rats, and not consistently (Fig. 6.2 and Fig. 6.22). Pregnant rats given NPY and naloxone spent least time grooming and drinking, consistent with their reduced eating behaviour (Fig. 6.22)

6.4.2. Changes in pregnancy in responses to centrally administered NPY of hypothalamic circuitry regulating HPA axis and eating behaviour

pPVN

The results show that i.c.v. NPY significantly increased the number of Fos positive cells in the pPVN of virgin rats compared with aCSF treated rats (Fig.6.3). After i.c.v. NPY there was also a significantly greater number of Fos positive cells in the pPVN in virgin rats compared to late pregnant rats (Fig.6.3). This indicates reduced responsiveness in late pregnancy of the CRH/AVP neurones to NPY as these neurones are found in large quantities in the pPVN (see HPA axis section below). In male rats i.c.v. NPY induces Fos expression in the pPVN (Li *et al,* 1994). NPY injection markedly increased Fos-like immunoreactivity in the medial subdivision of the PVN, and after food ingestion additional populations of neurones in the lateral subdivision of the pPVN were activated (Li *et al,* 1994).

mPVN and SON

I.c.v. NPY also significantly increased the number of Fos positive cells in the mPVN (Fig. 6.4) and SON (Fig. 6.8) in virgin rats compared to rats given aCSF, and significantly increased the number of Fos positive cells in the mPVN (Fig.6.4) and SON (Fig 6.8) in virgin rats compared to late pregnant rats. NPY has been shown to increase plasma oxytocin concentration in female rats, but is ineffective during late

pregnancy (Brunton *et al,* 2006). The results show that NPY significantly increased the percentage of oxytocin neurones expressing Fos in the SON (Fig. 6.10) and the mPVN (Fig. 6.7) in virgin rats given NPY compared to virgin controls and compared to pregnant rats given NPY. There was a greater percentage of oxytocin neurones activated in response to NPY in the SON compared to the mPVN in virgin rats. These results suggest that oxytocin neurones in the SON are more involved in the response to NPY than oxytocin neurones in the mPVN. Studies in male rats have shown that the number of Fos positive cells in the PVN in response to NPY increased in association with feeding (Yokosuka *et al,* 1998). This suggests a subpopulation of neurones in the mPVN containing NPY receptors may be involved in regulating feeding upon activation of the NPY input. Administration of a Y1 receptor antagonist suppressed the feeding response of NPY neurones (Yokusuka *et al,* 1999). The finding that oxytocin neurones are activated by i.c.v. NPY is consistent with previous findings (Brunton *et al,* 2006). In previous studies in male rats there is an increase in Fos-like immunoreactivity in the SON in rats that were allowed to eat, this may be due to osmotic stimulation; rats have been shown to drink after NPY administration (Clark *et al,* 1985), though this was not evident in the present study (Fig. 6.2 and Fig. 6.22). It has also been proposed that activation of the SON in response to NPY involves activation of oxytocin, vasopressin and CCK producing cells (CCK cells have also shown to be present in the SON) (Li *et al,* 1994). It has however also been proposed that a subset of oxytocin, vasopressin and CCK neurones respond to NPY only in conjunction with food consumption (Li & Rowland, 1994). In the present study, the rats were allowed access to food, and even so oxytocin neurones did not respond to NPY in late pregnant rats (Fig. 6.4, Fig. 6.10), despite stimulation of

eating behaviour and food intake (Fig. 6.2). Notably, Fos expression in oxytocin neurones is stimulated by α-MSH, which inhibits eating (Sabatier, 2006), as does central oxytocin administration (Brunton et al, 2006).

ARC

The present results show that NPY also significantly increased Fos expression in virgin rats in the ARC compared to virgin controls and compared to pregnant rats given NPY (Fig. 6.11). It has been shown that NPY induces Fos expression in the ARC in the area where Y1 receptor mRNA has been localised (Li *et al,* 1994). Diet restriction and starvation rapidly induce accumulation of NPY in the PVN by increasing NPY mRNA levels in the ARC (White & Kershaw, 1990). NPY mRNA levels in the ARC have been reported to be significantly increased during pregnancy (Garcia *et al,* 2000), and not to change (Wilding *et al,* 1997). Increased NPY gene expression during pregnancy may be part of the adaptive mechanisms present in the physiological state of pregnancy. Nothing is known about NPY receptor expression in the ARC during late pregnancy. It would be interesting to characterise the phenotype of the ARC neurones responding to NPY.

The lack of Fos activation in the ARC in late pregnant rats by i.c.v. NPY suggests that lack of excitation of a pool of ARC neurones might explain the lack of an HPA axis response, and indicates that ARC neurone excitation is not essential for eating responses to i.c.v. NPY.

LHA

The results show that NPY did not significantly increase the number of Fos positive cells in the LHA (the hunger centre) in either virgin or pregnant rats compared to control animals (Fig.6.13). It has previously been shown that Fos expression in the

LHA in response to NPY is very weak (Li *et al,* 1994). Y1 and Y5 receptors that are mainly involved in the food intake stimulatory activity of NPY are present in the LHA (Wolak *et al,* 2003) and NPY containing terminals innervate orexigenic neurones in the LHA. I.c.v. administration of NPY also increases Fos activity in the lateral hypothalamic orexigenic neurones of fasted rats (Campbell *et al,* 2003). Similarly rats that were given food showed no extra activation in the LHA where Fos expression had been shown to be weak following i.c.v. NPY (Li *et al,* 1994). Rats in the present study were not fasted before the experiment.

It is important to note that Fos expression indicates excitation of neurones (Windle *et al,* 2004), and any direct inhibitory actions of NPY are not revealed.

VMH

The present results also show that NPY did not significantly increase the number of Fos positive cells in the VMH (the satiety centre), although there was a slight tendency for the number of Fos positive cells to increase in the VMH in both virgin and pregnant rats after NPY (Fig.6.14). Again previous studies in male rats showed Fos expression to be weak following administration of NPY (Li *et al,* 1994) and these results did not change following food intake (Li *et al,* 1994). Injection of NPY into the VMH increases food intake (Morley *et al* 1987). NPY was found to inhibit one fifth of VMH neurones, and this inhibition was potentiated by overfeeding (Heidel *et al,* 1999).

DMH

The results showed that NPY did not significantly increase the number of Fos positive cells in the DMH (Fig.6.15). Pregnant rats given aCSF showed a significantly greater number of Fos positive cells compared to all other groups, which might be interpreted as showing an inhibitory action of NPY in late pregnancy. In previous studies with male rats a moderate Fos response was seen in response to NPY in the DMH and this was increased to strong with food intake (Li *et al,* 1994). Microinjection of NPY into the DMH results in stimulation of feeding (Corp *et al,* 1990). It is possible that this region may also contain NPY target cells involved in feeding behaviour.

Six receptor subtypes have been found to mediate the actions of NPY (Gerald *et al,* 1996). These are widely distributed throughout the brain. The PVN, SON and the ARC express the Y1 and Y5 receptor (Wolak *et al,* 2003). To date there is no evidence regarding NPY receptor expression in which could also explain changes in neuroendocrine responses seen in late pregnancy. It should also be reiterated that Fos expression allows detection of neurones that have been excited (Hoffman *et al,* 1993) and differences in inhibitory actions on pools of neurones would not be detected.

Overall, the above findings indicate, since feeding responses to NPY are intact in late pregnancy, that activation of the pPVN, magnocellular oxytocin neurones and ARC neurones is not essential for stimulation of feeding by NPY in late pregnancy. However, stimulation of these neurones may be important in the activation of the HPA axis by NPY (see below).

6.4.3. The effect of endogenous opioids on NPY-induced Fos expression in hypothalamic circuits

Previous results have shown that the reduced responsiveness of the CRH/AVP pPVN neurones to centrally administered NPY in late pregnancy (Brunton *et al,* 2006) could be due to enhanced inhibition by a central opioid mechanism that emerges in pregnancy. Possible sources of endogenous opioid that could regulate PVN CRH/AVP neurones include noradrenergic NTS neurones which coexpress pro-enkephalin-A (pENK-A) mRNA (Cecatelli *et al,* 1989). pENK-A mRNA expression is increased in the NTS in late pregnancy and may be the source of opioid inhibiting noradrenaline release onto CRH neurones following administration of interleukin-1β (Brunton *et al,* 2005). NPY-containing neurones project from the NTS to the PVN. It was not known previously whether the opioid mechanism would interfere with NPY signalling to pPVN CRH/AVP, mPVN, SON or ARC neurones which is shown here (see above) to be reduced during pregnancy.

pPVN

The present results show that NPY increased the number of Fos-positive cells in the pPVN in virgin rats compared to pregnant rats, consistent with previous experiments (Brunton *et al,* 2006). Naloxone given before NPY had no further effect on the number of Fos positive cells in virgin rats, but significantly increased the number of Fos positive cells in pregnant rats given NPY and naloxone compared to pregnant rats given NPY and saline (Fig.6.25). There was no significant difference between virgin rats given NPY and naloxone and pregnant rats given NPY and naloxone (Fig.6.25). This provides further evidence for the presence of an opioid inhibition on the CRH/AVP neurones in pregnancy, and that this extends to the actions of NPY.

mPVN

NPY significantly increased the number of Fos positive cells in the mPVN in virgin rats with no further changes following administration of naloxone. The number of Fos-positive cells was significantly lower in pregnant rats given NPY compared to virgin rats, but there was no further change in Fos expression following naloxone (Fig.6.27). However, pregnant rats given NPY and naloxone had a significantly greater number of Fos-positive cells compared to pregnant controls given naloxone alone, whereas pregnant rats given NPY and saline showed no significant difference from aCSF treated rats (Fig 6.27).This suggests that there is a weak degree of opioid inhibition on the mPVN neurone response to NPY during late pregnancy.

SON

NPY significantly increased the number of Fos-positive cells in the SON in virgin rats compared to virgin controls and compared to pregnant rats. Naloxone did not affect the SON neurone Fos response to NPY in virgin rats. The number of Fos positive cells in virgin rats given NPY and naloxone was not significantly different from the number of Fos positive cells in pregnant rats given NPY and naloxone. There was a significantly greater number of Fos positive cells in pregnant rats given NPY and naloxone compared to pregnant rats given aCSF and naloxone (Fig. 6.28); but again this suggests only a weak opioid inhibition on the SON neurone responses to NPY during late pregnancy, in contrast with previous studies, using other stimuli where there is strong opioid inhibition (Douglas *et al,* 1995; Russell and Brunton, 2005). It has been shown in response to a cytokine stressor that the failure of oxytocin neurones to respond in pregnant rats is a result of an opioid inhibition;

naloxone not only restored the oxytocin secretory response in pregnant rats but unmasked an exaggerated response (Russell & Brunton, 2005). Importantly, electrophysiological recording has shown that naloxone acts centrally to reverse the opioid inhibition on oxytocin neurone responses to interleukin-1β in late pregnancy (Brunton *et al.* 2005). It is important to note that further studies, involving measurement of oxytocin secretion and electrophysiological recording are required to resolve the issue of opioid involvement in suppressed oxytocin neurone responses to i.c.v. NPY in late pregnancy.

ARC, LHA, VMH, DMH

I.c.v. NPY significantly increased the number of Fos positive cells in the ARC in virgin rats compared to virgin controls with no significant difference seen following administration of naloxone. There were significantly more Fos positive cells in the ARC in virgin rats following NPY compared to pregnant rats; there was however a tendency for the number of Fos positive cells to increase after NPY in pregnant rats compared to pregnant aCSF-treated controls (Fig. 6.29). Naloxone had no effects and did not alter Fos expression in response to NPY in the LHA (Fig. 6.30), VMH (Fig.6.31) or DMH (Fig.6.32). Hence neither the restoration of HPA axis responses (Figs. 6.17, 18, 19, 25) nor the suppression of eating behavioural responses (Figs 6.22, 23, 24) to i.c.v. NPY in late pregnancy by naloxone is attributable to excitation of neurones in these areas.

6.4.4. The effect of i.c.v NPY and i.v. naloxone on blood glucose concentration

The results show that NPY significantly increased blood glucose concentration in virgin rats, with no change following administration of naloxone (Fig.6.16).

Within the brain, subgroups of neurones respond differently to altered blood glucose concentrations. Identification of NPY in hypothalamic neurones that sense glucose suggests a role for NPY in glucose sensing (Gozali *et al,* 2002). The reflex control of blood glucose is mediated by the NTS. The medulla receives sensory inputs from the stomach and portal vein which signals to the pancreas to release insulin. Insulin inhibits NPY release (Shwartz *et al,* 1996). Blood glucose can be an indicator of the overall metabolic response to NPY. Blood glucose concentration gives an indication of the balance between insulin, glucagon, growth hormone, corticosterone and sympathetic activity in the rat.

Basal blood glucose levels were significantly lower in pregnant rats (Fig.6.16). Previous studies have shown that basal blood glucose levels decline as pregnancy progresses (Rossi *et al,* 1993). NPY given i.c.v. did not significantly increase blood glucose in pregnant rats; naloxone did not alter this lack of effect of NPY in pregnant rats (Fig.6.16). Reduced responses in blood glucose during pregnancy could be due to reduced corticosterone secretion during pregnancy which has previously been shown (Brunton *et al,* 2006) or reduced noradrenergic responses during pregnancy which has been shown in response to other stressors (Douglas, 2005). As corticosterone responses are not evident until 30 minutes after administration of NPY (Brunton *et al,* 2006) the latter explanation is perhaps more probable as the increase in blood glucose is seen at 15 minutes. Furthermore, naloxone did not permit a hyperglycaemic response to i.c.v. NPY in late pregnant rats, indicating lack of corticosterone involvement since naloxone restored an ACTH response to NPY (see below). It would be interesting to measure the secretory profiles of the hormones mentioned above after i.c.v. NPY in late pregnant and virgin rats.

6.4.5. The effect of i.c.v. NPY and i.v. naloxone on the HPA axis

The results show that NPY significantly increased plasma ACTH secretion in virgin but not pregnant rats (Fig.6.17), consistent with previous studies (Brunton *et al,* 2006). Naloxone showed no further effect on plasma ACTH concentration in virgin rats, although the response appeared to be slightly more sustained which is consistent with other studies with different stressors looking at the effect of endogenous opioids (Brunton *et al,* 2005). Naloxone restored an ACTH response in pregnant rats (Fig.6.17, 18) indicating that opioids are interfering with NPY signalling to the HPA axis during late pregnancy. The results also show that NPY significantly increased CRH (Fig.6.19) and AVP mRNA expression (Fig.6.20) in the pPVN in virgin rats, but not in late pregnant rats; this has also been previously been shown for stressors (Brunton *et al,* 2006). Naloxone showed no further effect on pPVN CRH mRNA expression in virgin rats, yet restored a response to NPY in the pregnant rats (Fig.6.19). Similarly, naloxone showed no further effect on AVP mRNA expression in virgin rats after NPY although there was a tendency for AVP mRNA to be increased in virgin rats given NPY and naloxone compared to virgin rats given NPY and saline. Naloxone restored the pPVN AVP mRNA response to NPY in pregnant rats (Fig.6.20), further indicating that opioids are interfering with NPY signalling to the paraventricular CRH/AVP neurones in late pregnancy. Alternative mechanisms of suppressed oxytocin neurone responses to NPY in late pregnancy need to be considered. In late pregnancy the GABA input to the PVN and SON oxytocin neurones is more effective. Increased levels of allopregnanolone in the brain in pregnancy enhance the actions of GABA through actions at the $GABA_A$ receptor (Brussaard *et al,* 2000) and there are increased GABAergic synaptic contacts with

oxytocin neurones (Montagnese *et al,* 1987). Allopregnanolone restrains interleukin-1β stimulated ACTH secretion (Brunton *et al,* 2005) in late pregnancy, possibly through its actions on $GABA_A$ receptors. This mechanism may also stand for reduced oxytocin neurone (and HPA axis) responses in late pregnancy which would be interesting to investigate.

The many changes that occur in late pregnancy in the responses to central administration of NPY are summarised in table 6.2.

6.4.6. Summary

We have shown that in late pregnancy the increase in Fos expression is significantly reduced in the dorsomedial parvocellular division of the PVN, indicating reduced responses of the CRH/AVP expressing neurones, and confirming central suppression of HPA axis responsiveness in late pregnancy. Furthermore, naloxone partially reversed the attenuation of the HPA-axis responses to NPY in late pregnancy as shown by restoring pPVN CRH, AVP mRNA and ACTH, responses. We can conclude that endogenous opioids are evidently involved in suppressing HPA axis responses to NPY in late pregnancy.

Similarly there was a reduced Fos response in the SON and magnocellular PVN; specifically oxytocin neurones were not activated by NPY in pregnancy.

We have also shown there was no stimulation by NPY of Fos in the ARC in pregnancy, indicating another possible mechanism of reduced responsiveness of the HPA-axis in pregnancy. However, the finding that naloxone did not restore ARC responses indicates that ARC stimulation is not essential for NPY actions on the HPA axis. These findings are consistent with other studies demonstrating reduced

Brain area	Other	**i.c.v. NPY**	**i.c.v. NPY**	**i.c.v. NPY + NALOXONE**	**i.c.v. NPY + NALOXONE**
Fos in:		Virgin	Pregnant	Virgin	Pregnant
pPVN		++	*0	++	#+
mPVN		++	*(+)/0	++	#(+)
SON		++	*+)/0	+	#(+)
Oxytocin neurones		++	*0	-	-
ARC		++	*0	++	0
LHA		0	0	0	0
VMH		0	0	0	0
DMH		0	0	0	0
	Eating	++	++	+	#(+)/0
pPVN- CRH mRNA		++	*0	++	#++
pPVN- AVP mRNA		++	*0	++	#++
	Plasma ACTH	++	*0	++	#+
	Blood glucose	+	*0	+	0

Table 6.2: Summary of the central effects of NPY +/- naloxone in virgin and pregnant rats. pPVN = parvocellular PVN; mPVN = magnocellular PVN; SON = supraoptic nucleus; ARC = arcuate nucleus; LHA = lateral hypothalamic area; VMH = ventromedial hypothalamus; DMH = dorsomedial hypothalamus; CRH = corticotropin releasing hormone; AVP = arginine vasopressin; ACTH = adrenocorticotropic hormone. ++ = strongly stimulated; +: stimulated (+) = weakly stimulated; 0 = no stimulation; -: not done.
* significant difference vs. virgin; # significant effect of naloxone on response to NPY.

responsiveness of the HPA-axis and the neurohypophysial oxytocin system in response to various stressors in late pregnancy.

Naloxone partially restored a Fos response to NPY in the pPVN and SON, with a slight effect in the mPVN, These results indicate that opioids may indeed be interfering in late pregnancy with NPY signalling to the pPVN, but effects on NPY signalling to the mPVN and supraoptic neurones in pregnancy may not be important. Although Fos expression is not stimulated by i.c.v. NPY in certain hypothalamic areas eating responses to NPY remained intact in late pregnancy, except that naloxone attenuated the eating responses to NPY. It is possible then that NPY projections from the brainstem to the PVN are important in the orexigenic response to NPY. NPY did not induce Fos expression in the LHA, DMH or VMH in either virgin or pregnant rats. Fos expression remained significantly lower in pregnant rats in the ARC following naloxone and NPY, which indicates the lack of HPA axis activation after NPY in late pregnancy is not due to failure of ARC neurones that respond to NPY.

GENERAL DISCUSSION

7.1 General Discussion

Remarkable changes occur during pregnancy. These include increased appetite and reduced HPA axis responses during exposure to a stress (Brunton *et al,* 2006). The responsiveness of the HPA axis and the magnocellular oxytocin neurones to stressors is altered. This includes suppressed responses to both emotional and physical stressors in rats (Neumann *et al,* 1998) and mice (Douglas *et al,* 2003), in particular to immune challenge with IL-1β in rats (Brunton *et al,* 2005). The HPA axis is also hyporesponsive to the appetite regulating peptide orexin-A during late pregnancy (Brunton *et al,* 2003). This reduced responsiveness is known to be due to reduced responsiveness of the pPVN CRH and AVP neurones (Brunton *et al,* 2003). This may be a consequence of reduced neural drive or enhanced inhibitory inputs to these CRH neurones at the end of pregnancy. Evidently, enhanced negative feedback inhibition by corticosterone does not explain the hyporesponsiveness of the HPA axis to stress during late pregnancy (Johnstone *et al,* 2000). The attenuation of HPA axis responses to psychogenic stressors (Douglas *et al,* 1998) and IL-1β (Brunton *et al,* 2005) involves inhibition by a central endogenous opioid mechanism in pregnancy. There are several possible sources of endogenous opioid that restrain CRH neurones during late pregnancy including the ARC (Sawchenko *et al,* 1982), perifornical region and the NTS neurones (Ceccatelli *et al,* 1989). Suppression of HPA axis stress responses during late pregnancy protects the foetuses from excess glucocorticoids which can cause adverse programming. Suppression of HPA axis responses to centrally administered orexin-A during late pregnancy helps maintain a positive energy balance because glucocorticoids mobilise energy stores. Glucocorticoids stimulate both feeding and insulin secretion (Dallman *et al,* 1995). The relationship

between the HPA axis and factors relating to adipose tissue storage is very interesting. The aim of this thesis was to investigate changes in central mechanisms underlying reduced HPA responses to central neuropeptides signalling metabolic state during late pregnancy.

HPA axis responses to insulin-induced hypoglycaemia were maintained during late pregnancy which is in contrast to other stressors at this time. It is very interesting that this HPA axis activation is maintained when there is a severe energy lack, as opposed to a presumably more modest energy shortage signalled by orexin-A, to which the HPA axis is less responsive during late pregnancy. An interesting area for further investigation would be identification of the pathways involved in activating the various brain regions in response to insulin-induced hypoglycaemia. Insulin-induced hypoglycaemia may have stimulated ACTH secretion through AVP instead of CRH, which corresponds with similar studies where hypothalamo-hypophyseal portal blood concentrations of AVP were increased following insulin-induced hypoglycaemia with no changes in CRH (Plotsky *et al,* 1985). It seems that insulin-induced hypoglycaemia increases ACTH secretion by preferentially activating the pPVN AVP neurones – and very importantly the mechanism remains intact during pregnancy unlike responses to other metabolic signals such as orexin or NPY (Brunton *et al,* 2003 and 2006). Both of these signals activate the HPA axis in non-pregnant rats through stimulation of the pPVN CRH and AVP neurones. Ghrelin has been shown to activate the HPA axis in a similar way through NPY, so reduced action of NPY on the HPA axis in late pregnancy may underlie the reduced responses to orexin and ghrelin.

The mechanisms of ACTH regulation by ghrelin have been proposed to include hypothalamic release of CRH, AVP and NPY. The ARC NPY neurones are a likely target as they express the GHS-R. Ghrelin did not activate the HPA axis during late pregnancy, but the mechanisms of why ghrelin does not stimulate ACTH secretion during late pregnancy are not known although several possibilities exist. It has previously been shown that in response to orexin A reduced activation of the HPA axis is due to reduced stimulation of the pPVN CRH/AVP neurones, and a similar explanation was confirmed for reduced HPA responses to i.c.v. ghrelin. I.c.v. ghrelin increased *c-fos* expression in the pPVN in virgin rats, which corresponds with previous studies (Hu *et al,* 2005) yet was not activated in pregnant rats. Reduced responsiveness of the CRH and AVP neurones was also shown following i.c.v. injection of orexin or NPY where the HPA axis response was also reduced during late pregnancy, which also corresponds with previous studies (Brunton *et al,* 2003; 2006). A central opioid mechanism that emerges during late pregnancy has been shown to be responsible for reduced HPA responses to psychogenic stressors and immune challenge. The primary role of the PVN CRH neurones in response to challenge with IL-1β involves direct nor-adrenergic projections from the NTS in the brain stem; naloxone (an opioid receptor antagonist) triggers a pPVN CRH mRNA response to IL-1β indicating a role for endogenous opioids in restraining CRH neurones during late pregnancy (Brunton *et al,* 2005). It was not known if opioids would be involved in restraining CRH neurones in response to metabolic signals such as ghrelin, orexin and NPY. It was shown that the HPA axis was inhibited by endogenous opioids in late pregnant rats following administration of orexin and NPY, since after i.v. naloxone ACTH responses were restored following i.c.v.

injection of either i.c.v. NPY or orexin; this was also shown with *in-situ* hybridisation where naloxone increased both CRH and AVP mRNA following i.c.v. NPY. Further evidence for the presence of an opioid inhibition on the CRH/AVP neurones was shown when a Fos response was restored after i.c.v. NPY and i.v. naloxone. The same Fos study suggested there was a weak degree of opioid inhibition on the mPVN. Endogenous opioids are evidently involved then in suppressing HPA axis responses to metabolic signals in pregnancy.

7.2 What causes the changes in pregnancy?

These studies indicate that activation of inhibitory opioid mechanisms in the brain during pregnancy underlie the suppressed action of these orexigenic peptides on the HPA axis, and other studies indicate a similar mechanism restrains oxytocin neurones (Douglas…..; Russell and Brunton 2006). It has been hypothesised that pregnancy is signalled to the brain to induce the mechanisms that suppress appetite peptide action on stress circuits by neurosteroids produced from progesterone (Russell and Brunton 2006). Neurosteroids accumulate in the brain independently of the levels in peripheral tissues (Hu *et al,* 1987) and systemic administration of progesterone or its metabolite allopregnanolone (AP) induces various anxiolytic effects by enhancing the function of $GABA_A$ receptors (Majewska, 1992). Both of these findings indicate that changes in plasma and brain concentrations of progesterone or allopregnanolone may control neuronal excitability, through enhancing the function of $GABA_A$ receptors, by increasing the opening time of the chloride channel in the $GABA_A$ receptor (Bitran *et al,* 1995). There are more GABA synapses on oxytocin neurones during late pregnancy (Montagnese *et al,* 1987)

indicating enhanced action through $GABA_A$ receptors at this time. AP content in the brain is increased during late pregnancy because of increased secretion of progesterone (Luisi *et al,* 2000); the brain itself can metabolise progesterone to AP via 5α-reductase (Russell & Brunton, 2005). In late pregnant rats given the 5α-reductase inhibitor finasteride, to reduce AP levels to virgin levels, oxytocin responses after IL-1β were restored; additional treatment with naloxone did not further alter this response (Russell & Brunton, 2006). It has been proposed that increased AP could act by inducing opioid mechanisms during late pregnancy.

7.3 What is it all for?

Non stress levels of glucocorticoids play a key physiological role in promoting and maintaining a positive energy balance (Syed & Weaver, 2005). Glucocorticoids stimulate feeding and insulin secretion (Dallman *et al,* 1985). Pregnancy is a hypermetabolic state with a great increase in maternal body fat and weight, there is no increase in energy efficiency and energy balance becomes positive primarily because of an increase in food intake and this prevents depletion of maternal energy stores (Richard & Trayhurn, 1985). In the rat food intake increases by about 50% and food consumption usually occurs in the dark phase only (Booth & Nicholls, 1974). As described in this thesis several hypothalamic peptides have been identified as possible regulators of food intake and metabolism e.g. NPY, orexin and ghrelin. Diet restriction and starvation increase NPY in the PVN by increased NPY mRNA expression in the ARC. NPY mRNA levels in the ARC have previously been shown to be increased during late pregnancy (Garcia *et al,* 2003). This is surprising due to high leptin levels during late pregnancy, however it has been shown there is leptin

resistance in late pregnant rats (Garcia *et al,* 2000). In food-restricted late pregnant rats there is also an increase in circulating ghrelin levels (Guallio *et al,* 2002). None of the rats used in this thesis were food restricted, and ghrelin was administered centrally since this has previously been shown to increase NPY mRNA in the hypothalamus (Seone *et al,* 2003). Whether ghrelin still increases NPY mRNA in the ARC in late pregnant rats is not yet known. Surprisingly, hypothalamic expression of orexin has been shown to be reduced during late pregnancy (Garcia *et al,* 2002). Food restriction does not increase orexin mRNA levels as has previously been shown in virgin rats (Lopez *et al,* 2000). It is possible that there is inhibition of prepro-orexin expression in the hypothalamus that counteracts the stimulatory effect of low leptin levels in fasted pregnant rats (Garcia *et al,* 2002). This also indicates that orexin may not be involved in the regulation of increased food intake during pregnancy. Orexin is also related to arousal and sleep, and pregnancy is also associated with changes in sleep patterns (Santiago *et al,* 2001). It seems there are complex neural pathways involved in the mechanisms established during late pregnancy. Increased NPY gene expression may be part of the adaptive mechanisms; it is interesting that insulin-induced hypoglycaemia still stimulates ACTH responses during late pregnancy. Overall during late pregnancy reduced HPA axis and specifically corticosterone responses after i.c.v. ghrelin, orexin and NPY may reflect a switch to anabolic metabolism which would help meet the demands of the foetus without causing a negative energy balance in the pregnant mother. These responses are suppressed by endogenous opioids. Endogenous opioids also facilitate NPY-induced feeding in both virgin and late pregnant rats but are evidently more important in late pregnant rats, when central opioid mechanisms are up-regulated.

Activation of endogenous opioid mechanisms in pregnancy have opposite effects on neuroendocrine stress responses and appetite regulation.

7.4 Future plans

Since it is not known if it is insulin or hypoglycaemia that activates the HPA axis after acute bolus insulin injection, it would be interesting to see if insulin injection, whilst maintaining blood glucose by infusing glucose i.v., would still activate the HPA axis in virgin and pregnant rats.

Since increased AP in the brain during late pregnancy could also play a role in inducing the opioid mechanisms in pregnancy that suppress the orexigenic peptide actions on stress circuits, administration of finasteride to reduce AP production from progesterone levels would be an interesting experiment. The effects of centrally administered orexigenic peptides after finasteride, measured as plasma ACTH concentration, AVP and CRH mRNA and Fos expression changes in virgin and pregnant rats would be interesting to investigate.

Y1 and Y5 receptors have been identified in the PVN (Wolak *et al,* 2003). Y2 receptors are thought to play a key role in activating the HPA axis (Small *et al,* 1997). To date there are no reports about NPY receptor expression during late pregnancy. *In situ* hybridisation in virgin and pregnant rats to measure expression of Y receptor mRNAs would answer whether reduced expression might contribute to reduced NPY action on the HPA axis.

Neuroendocrine stress responses to centrally administered NPY are suppressed in late pregnant rats but ingestive behavioural responses remain intact. Double labelling immunohistochemical studies would identify the phenotype of neurones stimulated

by ghrelin, orexin and NPY and prove a useful way for further dissecting the pathways that are essential in the feeding response to NPY.

NPY-containing catecholaminergic neurones in the medulla project to the PVN (Sawchenko *et al,* 1985). Orexin (Willie *et al,* 2001) and ghrelin (Wynne *et al,* 2002) neurones also have projections to the NTS. Fos immunohistochemistry on brain stem sections from rats given ghrelin, orexin or NPY would indicate if the brain stem circuitry is activated in response to these orexigenic peptides.

A pilot study of Fos immunohistochemistry has begun in insulin-treated virgin rats to see the pattern of activation in the hypothalamus in these animals. Preliminary results showed activation in various areas involved in regulation of eating and in activating the HPA axis. Further experiments with more virgin animals and also in pregnant animals are needed.

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APPENDIX

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Appendix A

Publications

Brunton PJ, Bales J, Russell JA. Neuroendocrine Stress But Not Feeding Responses to Centrally Administered Neuropeptide Y Are Suppressed in Pregnant Rats. Endocrinology 2006 **147:** 3739-3745

Abstracts presented at British meetings

Douglas AJ, Briam V, Bales J. Stress hyporesponsiveness during pregnancy and parturition: sympathetic and adrenal medulla responses in rodents. Poster presentation at the 195th Meeting of the Society for Endocrinology, London, England, **November 1-3rd 2004.**

Bales J, Towers KL, Brunton PJ, Russell JA. ACTH responses to central ghrelin administration are reduced in pregnant rats. Oral Communication at the 2004 Annual Meeting of the British Society for Neuroendocrinology, Glasgow, Scotland, **September 6-8th 2004**

Bales J, Searle AR, Brunton PJ, Russell JA. Opioid inhibition of hypothalamo-pituitary-adrenal (HPA) axis responses to orexin-A during pregnancy. Poster presentation at the 2005 Annual Meeting of the British Society for Neuroendocrinology, Oxford, England, **September 4-6th 2005.**

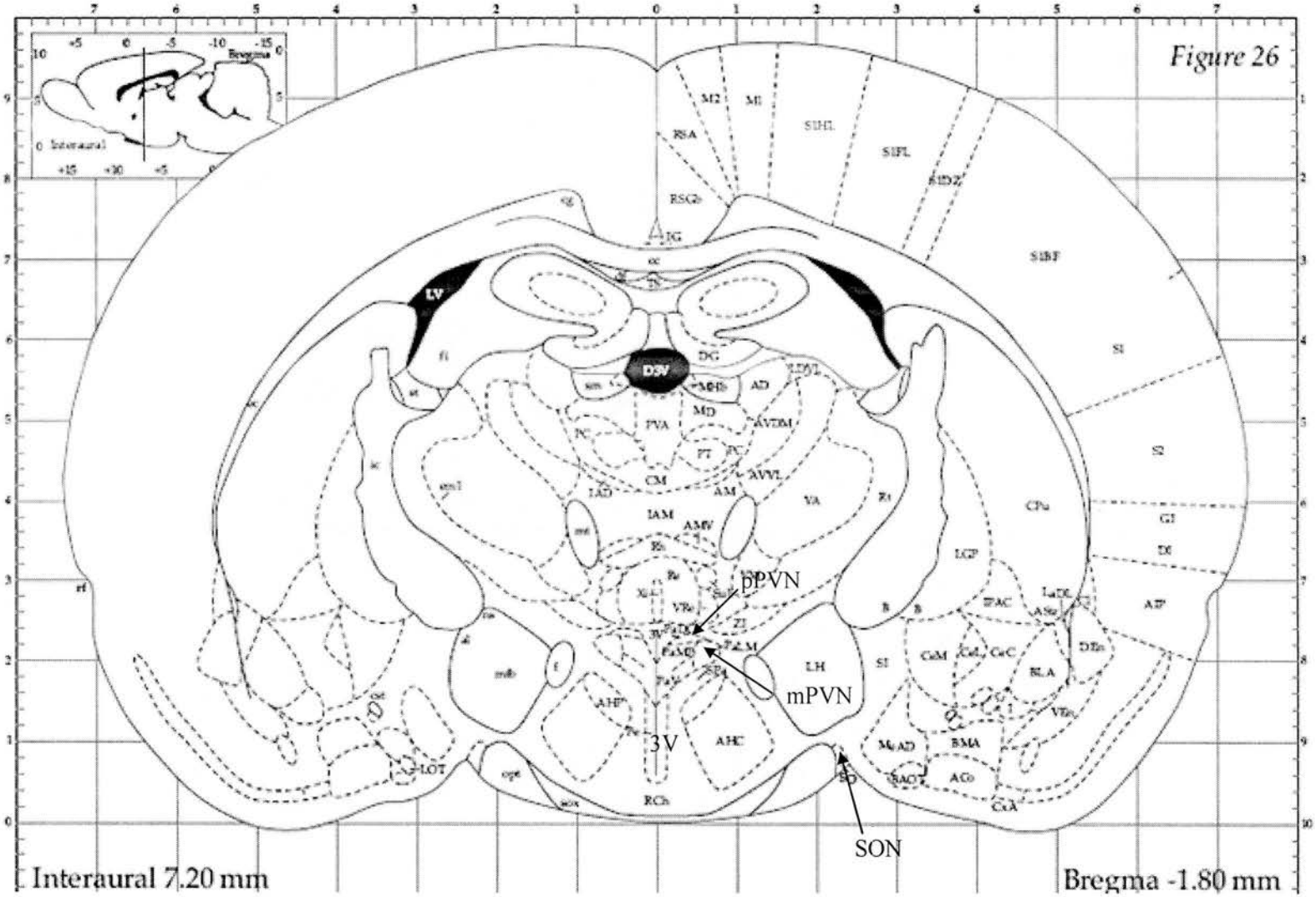
J.Bales, A.R.Searle, P.J.Brunton and J.A.Russell. Opioid inhibition of hypothalamo-pituitary-adrenal (HPA) axis responses to neuropeptide Y (NPY) during pregnancy. Oral communication at the 196th Meeting of the Society for Endocrinology, London, England, **November 7-9th 2005.**

Abstracts presented at International meetings

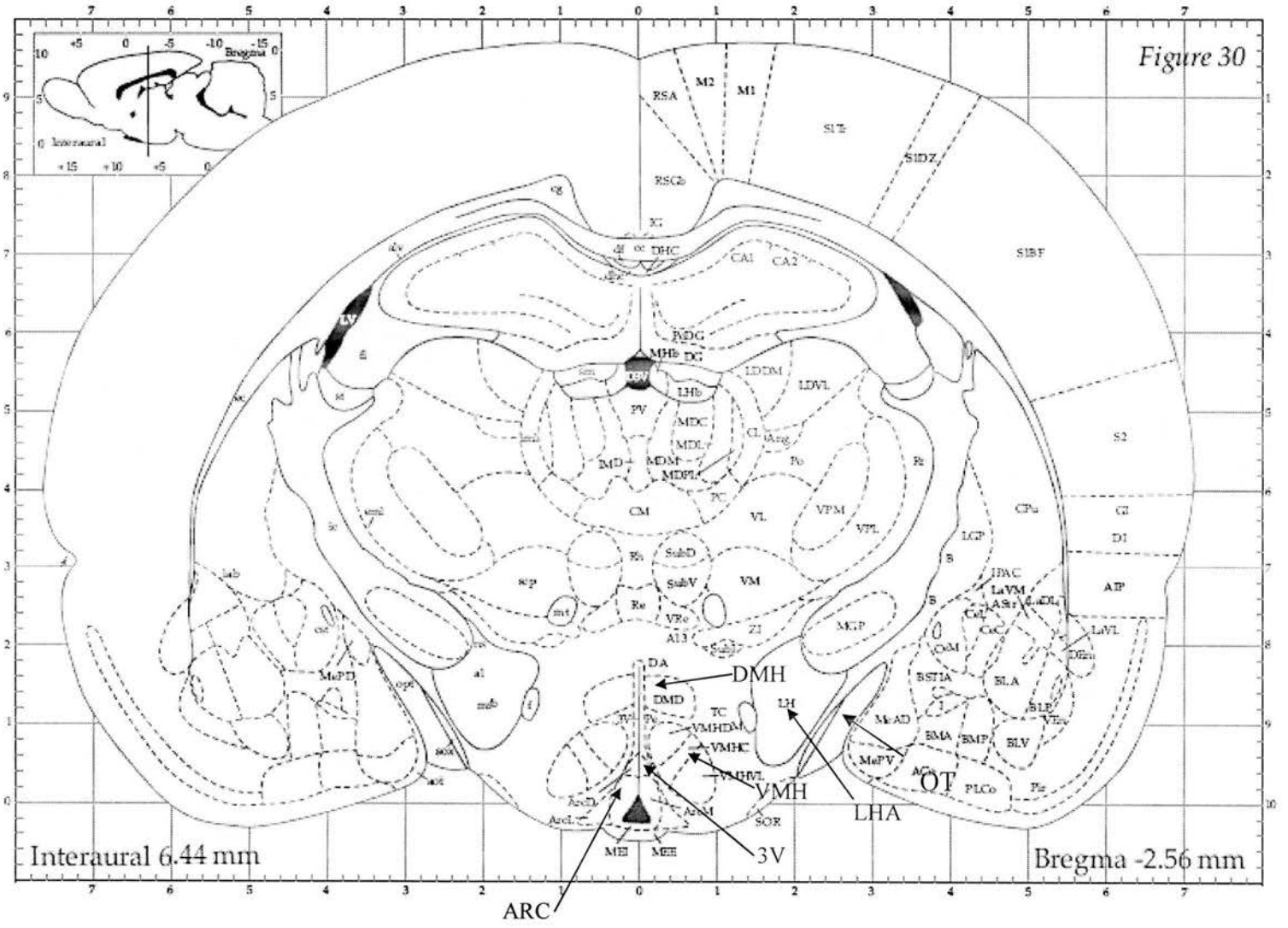
Bales J, Brunton PJ, Russell JA. Responses of the hypothalamo-pituitary-adrenal (HPA) axis to central ghrelin and systemic insulin administration during late pregnancy. Poster presentation at the Sixth International Congress of Neuroendocrinology, Pittsburgh, USA. **June 19-22nd 2006.**

Bales J, Brunton PJ, Russell JA. Suppressed hypothalamo-pituitary-adrenal (HPA) responses to central neuropeptide Y (NPY) in late pregnancy and the role of endogenous opioids. Poster presentation at the Sixth International Congress of Neuroendocrinology, Pittsburgh, USA. **June 19-22nd 2006.**

Bales J, Brunton PJ, Russell JA. Endogenous opioids and Neuropeptide Y (NPY) – induced feeding in pregnant rat. Poster presentation at the Society for Behavioural Neuroendocrinology 10th Annual meeting, Pittsburgh, USA. **June 17-20th 2006.**



Appendix B: A coronal section of the rat brain showing the parvocellular paraventricular nucleus (pPVN), magnocellular paraventricular nucleus (mPVN) and supraoptic nucleus (SON). 3V = 3rd ventricle. Taken from The Rat Brain in stereotaxic coordinates, 4th Edition, George Paxinos and Charles Watson



Appendix C: a coronal section of the rat brain showing the arcuate nucleus (ARC), the ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) and the lateral hypothalamic area (LHA). 3V = 3rd ventricle, OT = optic tract. Taken from The Rat Brain in stereotaxic coordinates, 4th Edition, George Paxinos and Charles Watson